REMARKS

I. Introductory Remarks

Applicants request reconsideration of this application in view of the following remarks.

Claims 22-23, 25-34 and 36-44 remain pending in this application, with claim 44 being withdrawn from consideration. No claims presently are being amended, added or canceled.

II. The Claims Comply with the Written Description Requirement of 35 U.S.C. § 112

The Office rejected claims 22, 25-33 and 36-43 as allegedly failing to comply with the written description requirement. According to the Office, "[i]n order to form an antigen binding structure, an antibody-type polypeptide requires the participation of both the heavy and light chain variable regions." The rejected claims specify the structure of a V_H or V_L domain, but do not specify the structure of a complementary V_L or V_H domain, respectively. The Office alleged this to be improper, because the specification describes antigen binding structures that have defined V_H domains and defined V_L domains. Applicants respectfully traverse the rejection.

The rejection is based on a false premise that antigen binding structures require both heavy and light chain variable regions. However, the scientific literature up to 1996 contained many reports of antigen binding structures that had less than two complete variable domains, including the following:

- Levi *et al.*, PNAS (USA), 90: 4374-4378 (1993) (copy enclosed) reported that a synthetic peptide containing a single CDR of a mouse monoclonal antibody specifically bound to a neutralization-inducing region of HIV-1, and showed potent antiviral activity.
- Monfardini *et al.*, J. Biol. Chem., 270(12): 6628-6638 (1995) (copy enclosed) reported the development of a recombinant light chain library in *E. coli* derived from mice immunized with polyclonal anti-GM-CSF. Several clones bound to an anti-GM-CSF monoclonal antibody, and one inhibited immunoprecipitation of the monoclonal antibody.
- Williams *et al.*, J. Biol. Chem., 266(8): 5182-5190 (1991) (copy enclosed) reported the development of conformationally constrained peptides, based on the V_L domain of

a neutralizing anti-reovirus type 3 monoclonal antibody, that have high binding affinity. The paper also describes earlier work in which linear peptides based on the V_L and V_H domains of the same antibody bound to targets.

- Ward *et al.*, Nature, 341: 544-546 (1989) (copy of abstract enclosed) reported the recombinant production of V_H domains having antigen binding activities against two different antigens, lysozyme and keyhole limpet hemocyanin. The anti-lysozyme V_H domains were shown to have high and specific antigen-binding affinities. This work was reviewed in Ward, FASEB J., 6: 2422-2427 (1992) (copy enclosed), which further noted that V_H domains with specificities against mucin and influenza virus neuraminidase subsequently were isolated.

Thus, those skilled in the relevant art understood in 1996 that specific antigen binding does not require both the V_L and V_H domains of an antibody.

In order to satisfy the written description requirement, a patent specification need not provide literal support for the claimed subject matter. *See Fujikawa v. Wattanasin*, 93, F.3d 1559, 1570, 39 USPQ2d 1895, 1904 (Fed. Cir. 1996). A patent specification is directed to one or ordinary skill in the art. *Wang Laboratories, Inc. v. Toshiba Corp.*, 993 F.2d 858, 26 USPQ2d 1767 (Fed. Cir. 1993). Thus, "[i]f a person of ordinary skill in the art would have understood the inventor to be in possession of the claimed invention at the time of filing, even if [not] every nuance of the claims is explicitly described in the specification, then the adequate written description requirement is met." *In re Alton*, 76 F.3d 1168, 37 USPQ2d 1578 (Fed. Cir. 1996).

"[T]hat a claim may be broader than the specific embodiment disclosed in a specification is in itself of no moment." (*In re Peters*, 723, F.2d 891, 221 USPQ 952 (Fed. Cir. 1983), quoting *In re Rasmussen*, 650 F.2d 1212, 211 USPQ 323 (Cust. & Pat. App. 1981)). The Office has not identified any language in the specification that limits "antigen binding structures with specificity for Rhesus D antigens" to structures that comprise both a specified V_L domain and a specified V_H domain. Moreover, as explained above, skilled artisans in 1996 understood that antigen binding structures could function with only one V_L or V_H domain. Without any language in the specification otherwise limiting the invention, therefore, skilled

artisans would have understood the invention to include antigen binding structures that contain only one of the specified V_L or V_H domains.

Thus, the claims comply with the written description requirement, and Applicants respectfully request withdrawal of the rejection.

III. The Claims Comply with the Enablement Requirement of 35 U.S.C. § 112

The Office also rejected claims 22, 25-33 and 36-43 as allegedly failing to comply with the enablement requirement. Again, the Office based this rejection on the false premise that "[i]n order to form an antigen binding structure, an antibody-type polypeptide requires the participation of both the heavy and light chain variable regions." The rejected claims specify the structure of a V_H or V_L domain, but do not specify the structure of a complementary V_L or V_H domain, respectively. The Office alleged this to be improper, because an artisan allegedly "would not be able to determine the structure of and construct a heavy or light chain variable region polypeptide that binds Rhesus D antigen when used in conjunction with one of the listed heavy or light chain regions enumerated in the specification." Applicants respectfully traverse the rejection.

As explained above, the scientific literature up to 1996 contained many reports of antigen binding structures that had only one variable region or even less than one complete variable region. Thus, one skilled in the art in 1996 could have constructed, without undue experimentation, useful antigen binding structures that comprise one of the complete V_L or V_H domains specified in the claims, in combination with a complementary V_L or V_H domain.

Thus, the claims comply with the enablement requirement, and Applicants respectfully request withdrawal of the rejection.

IV. Concluding Remarks

Applicants request favorable reconsideration of this application, which now is in condition for allowance. If the Examiner believes that an interview would advance prosecution, he is invited to contact the undersigned attorney by telephone.

The Commissioner is hereby authorized to charge any additional fees that may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicants hereby petition for such extension under 37 C.F.R. § 1.136 and authorizes payment of any such extension fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date 27 May 2005

By _____

FOLEY & LARDNER LLP
Washington Harbour
3000 K Street, N.W., Suite 500
Washington, D.C. 20007-5143
Telephone: (202) 672-5475
Facsimile: (202) 672-5399

Beth A. Burrous
Attorney for Applicants
Registration No. 35,087

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Immunology

A complementarity-determining region synthetic peptide acts as a miniantibody and neutralizes human immunodeficiency virus type 1 *in vitro*

MICHAEL LEVI*, MATTI SÄLLBERG*, ULLA RUDÉN*, DOROTHEE HERLYN†, HARUHIKO MARUYAMA†, HANS WIGZELL‡, JAMES MARKS§¶, AND BRITTA WAHREN*‖

*Department of Virology, National Bacteriological Laboratory, 105 21 Stockholm, Sweden; †The Wistar Institute, 36th Street at Spruce, Philadelphia, PA 19104-4268; ‡Department of Immunology, Karolinska Institute, 104 01 Stockholm, Sweden; and §Cambridge Centre for Protein Engineering, Cambridge, CB2 2QH England

Communicated by Sune Bergström, February 11, 1993

ABSTRACT **A complementarity-determining region (CDR) of the mouse monoclonal antibody (mAb) F58 was constructed with specificity to a neutralization-inducing region of human immunodeficiency virus type 1 (HIV-1). The mAb has its major reactivity to the amino acid sequence I—GPGRA in the V3 viral envelope region. All CDRs including several framework amino acids were synthesized from the sequence deduced by cloning and sequencing mAb F58 heavy- and light-chain variable domains. Peptides derived from the third heavy-chain domain (CDR-H3) alone or in combination with the other CDR sequences competed with F58 mAb for the V3 region. The CDR-H3 peptide was chemically modified by cyclization and then inhibited HIV-1 replication as well as syncytium formation by infected cells. Both the homologous IIIB viral strain to which the F58 mAb was induced and the heterologous SF2 strain were inhibited. This synthetic peptide had unexpectedly potent antiviral activity and may be a potential tool for treatment of HIV-infected persons.**

Neutralizing antibodies play a crucial role in the human immune defence against the human immunodeficiency virus (HIV). There are different opinions as to the capacity of antibodies alone to prevent infection by HIV. Undoubtedly, they are important therapeutic tools during the asymptomatic period following infection. Passive immunization with neutralizing antibodies may protect from virus challenge or decrease the replication of HIV (1, 2). Eventually, the disease progresses to acquired immunodeficiency syndrome (AIDS) as new virus strains appear that escape neutralization. However, neutralizing antibodies that bind conserved amino acid sequences common to many divergent virus strains might participate to delay development of AIDS.

B-cell maturation is characterized by somatic hypermutation in antibody variable region (V) genes and selection of B cells expressing high-affinity variants of this antigen binding site (3). The mouse monoclonal antibody (mAb) F58 binds to the conserved I—GPGR sequence of V3, which is the major neutralization-inducing region of the HIV-1 outer envelope glycoprotein gp120 (4, 5). Neutralizing antibodies against the V3 top region most probably act by blocking a postbinding interaction between V3 and the host cellular membrane necessary for viral entry (6, 7). The GPGR sequence is common to most western HIV-1 virus strains (8) and F58 has been shown to have both a strong type-specific neutralization capacity as well as cross-reactive properties (4).

Complementarity-determining regions (CDRs) are hypervariable loop structures in the antigen-binding parts of antibodies and determine the specificity of antigen binding (9). Areas of amino acid hypervariability within the antigen-binding region of an antibody correspond to six loop regions, of which five have an internal canonical structure. The sixth region, the heavy (H)-chain CDR3, is more variable in both amino acid sequence and length (10). These regions combine to form an antigen-contacting groove, stabilized by a β-barrel framework. Improvement of antibody affinity may be obtained by site-directed mutagenesis of individual amino acid residues in the antibody CDRs (11). The functional binding to antigen by an antibody is strongly conformation dependent on proximity and collaboration between amino acids of these regions. Nevertheless, binding or competition by peptide sequences from selected CDRs of mAbs has recently been demonstrated (12, 13). The aim of this study was to identify the CDR region(s) of mAb F58 responsible for interacting with the HIV-1 V3 region. We also wanted to study whether specific CDR(s) could neutralize HIV-1 *in vitro*.

MATERIALS AND METHODS

Cloning of mAb F58 V_H and V_κ Genes. The V domains of the κ light and H chains of the HIV-1-neutralizing mouse mAb F58 were cloned and sequenced. Briefly, first-strand cDNA was synthesized from $\approx 10^7$ hybridoma cells by a boiling method (14). The V_H and V_κ genes were amplified from aliquots of first-strand cDNA by PCR and cloned into the vectors M13VHPCR1 and M13VκPCR1 (15). At least two clones from two separate amplifications of the V_H and V_κ genes were sequenced. The deduced amino acid sequences of the V domains were used to synthesize CDR-based peptides.

Peptides and Proteins. V3 top regions derived from four HIV strains (IIIB, MN, RF, and SF2), all CDR regions (see Table 1), and all combinations of CDRs were prepared as synthetic peptides. The combinations were prepared as continuous sequences 29–33 amino acids long. The cysteines introduced in peptides V3-MN and CDR-H3/C1–C4 were chemically oxidized to create loop structures. Synthesis was performed according to Merrifield with modifications (16–18). The recombinantly produced envelope precursor protein of HIV-1 (rgp160) was kindly donated by Frank Volvovitz (MicroGeneSys, Meriden, CT). The rgp160 was derived from the $HIV\text{-}1_{IIIB}$ strain and was produced in cells of lepidopteran insects by using a baculovirus expression system.

Epitope Blocking by CDRs. Ninety-six-well enzyme immunoassay (EIA) plates (Nunc, Denmark) were coated with V3 peptides (1 μg per well) or rgp160 (0.1 μg per well) in 100 μl

Abbreviations: CDR, complementarity-determining region; HIV, human immunodeficiency virus; mAb, monoclonal antibody; EIA, enzyme immunoassay; V, variable region; H chain, heavy chain; rgp160, recombinantly produced envelope precursor protein of HIV-1.

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¶Present address: Department of Anesthesia, San Francisco General Hospital, University of California, CA 94110.
‖To whom reprint requests should be addressed.

of 0.05 M sodium carbonate buffer (pH 9.5) at room temperature overnight and stored at least 24 h at +4°C until use. CDR peptides were diluted in phosphate-buffered saline with 0.5% bovine serum albumin/0.05% Tween 20/0.01% merthiolate/2% goat serum. Each CDR peptide at 0.4–10 μg per 100 μl was added to the wells and incubated for 1 h at 37°C. An unrelated peptide from the N-terminal end of the HIV-1 envelope protein gp41 was included as a negative control. Then mAb F58 was added at 20–50 ng/ml and incubated for 1 h at 37°C. After washings, peroxidase-conjugated rabbit anti-mouse IgG (Dakopatts, Denmark) was added and incubated for 1 h at 37°C. Finally, the substrate orthophenylenediamine was added for 30 min at 37°C and the reaction was stopped by adding 2.5 M H_2SO_4. The absorbance was measured at 490 nm. A 50% decrease of mAb F58 binding was considered significant. In a modification of the assays described above, 10 ng to 10 μg of CDR peptide and 2 ng to 50 μg of mAb F58 were simultaneously added to EIA wells coated with V3 peptide or rgp160. Binding of mAb F58 was indicated as described above. As a control experiment, the CDR peptides were also assayed for inhibition of binding between the anti-HBe/β mouse mAb 57/8 and its hepatitis B epitope HBe 128–133 (19). In each blocking and competition assay, the mAb F58 was first titrated for binding to coated V3 peptide or gp160 to give absorbance values between 0.2 and 1.0.

mAb F58 Binding by Anti-Idiotypes. To generate anti-idiotypic mAbs against mAb F58, BALB/c mice (Harlan–Sprague–Dawley) were immunized subcutaneously with 20 μg of mAb F58 $F(ab')_2$ per injection coupled to keyhole limpet hemocyanin. The number of injections and the adjuvant used varied for the various anti-idiotypes produced [anti-idiotypes MF3H12, MF2B10, and MG2H3: first immunization in complete Freund's adjuvant, second to sixth immunizations in incomplete Freund's adjuvant; anti-idiotypes ML5H10, MN4A7, MN5B11, and MM5E1: one immunization in Titer Max adjuvant (CytRX, Norcross, GA)]. All mice seropositive for anti-idiotypic antibodies were injected intravenously with 20 μg of F58 $F(ab')_2$ (anti-idiotype MG2H3) or F58 $F(ab')_2$ keyhole limpet hemocyanin (all other anti-idiotypes) 3 days before fusion of splenocytes to SP2/0 mouse myeloma cells following standard protocols using polyethylene glycol 1500 and hypoxanthine/aminopterin/thymidine-containing selection medium. Hybridoma culture supernatants diluted 10^{-1}–10^{-5} were incubated with mAb F58 for 1 h at 37°C. This mixture was added to the wells of a V3-IIIB-coated EIA plate and incubated for 1 h at 37°C. The following procedures were the same as described above. Tissue culture supernatants were also tested for binding to the six CDRs of mAb F58 using EIA plates coated with 1 μg of CDR peptide per well. Supernatants were used in dilutions of 10^{-2} and 10^{-3}.

***In Vitro* HIV-1 Neutralization.** CDR peptides (50 ng to 50 μg) were incubated with HIV-1_{IIIB} or HIV-1_{SF2} in 100 μl per EIA well for 1 h at 37°C. Each peptide was tested in duplicate, 50 ng to 50 μg per well of F58 was included as a positive control, and 10^4 cells of human $CD4^+$ T-cell line C8166 were added to each well and incubated for 1 h at 37°C in 200 μl per well. The cells were washed once in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum and then incubated in 200 μl of medium per well for 6 days at 37°C and 5% CO_2/95% air. After 6 days, the neutralizing capacity of the CDR peptides was determined by measuring HIV-1 p24 antigen content in cell culture supernatants by capture EIA, by microscopic evaluation of syncytia, and by immunofluorescence. A 50% reduction of p24 content or of the number of syncytia was defined as neutralization (IC_{50}). Fifty micrograms of each CDR peptide was incubated with cells for 1 h at 37°C to detect possible toxic effects. Trypan blue exclusion and the number of cells were evaluated after 6 days.

RESULTS

Epitope Blocking. Peptides based on CDR1 of the V_κ domain and CDR2 of the V_H domain of mAb F58 included only the CDR sequence. The other CDR peptides included neighboring framework residues in addition to the CDR residues (Table 1). These additions were made to obtain peptides of similar lengths. The CDR peptides were used to directly block the V3 epitope of HIV-1 represented by peptide V3-IIIB. Only the peptide CDR-H3, representing the third H-chain CDR of mAb F58, and its combinations with sequences representing the other five CDR regions could block the binding of mAb to V3-IIIB (Fig. 1*A*). The best inhibition was obtained with the combined peptide representing CDR-H2/H3.

Competition Between mAb F58 and CDRs. CDR peptides and mAb F58 were allowed to compete for binding to viral antigens represented by recombinant gp160 or V3 peptides. CDR-H3 and all its combinations competed with the mAb for binding to the antigens (data not shown). CDR-H3 and its cyclic analogues (Table 1) competed well with mAb F58 for gp160 epitopes (Fig. 1*B*) and heterologous V3 sequences represented by peptides V3-MN, V3-RF, and V3-SF2 (Fig. 1*C*). Interestingly, CDR-H1 and CDR-H2 by themselves instead enhanced binding of mAb F58 to gp160 (Fig. 1*B*), indicating their independent interaction with the V3 region. All cyclic CDR-H3 peptides competed with mAb F58 better than the linear CDR-H3 (Fig. 1 *B* and *C*). None of our anti-HIV mAb F58-based CDR peptides could inhibit binding between the anti-HBe/β mAb 57/8 and its epitope, verifying the specificity of our competition results.

Inhibition of mAb F58 by Anti-Idiotypes. Mouse anti-idiotypic antibodies bound exclusively to mAb F58 but not to normal mouse IgG (results not shown). The anti-idiotypes were used to inhibit the mAb F58 binding to peptide V3-IIIB. Six of seven different anti-idiotypic hybridoma culture supernatants showed >50% inhibition at a dilution of 10^{-2}. To determine whether the inhibition was caused by direct binding of the anti-idiotypic antibodies to antigen-interacting regions of mAb F58, we assayed the supernatants for binding to the six CDR peptides. No binding of any of the seven anti-idiotypes to any of the CDRs could be detected.

Inhibition of HIV-1 Replication. CDR peptides (0.5 μg to 0.5 mg/ml) were used to neutralize or inhibit syncytium formation by HIV-1_{IIIB} or HIV-1_{SF2}. The cyclic forms of the CDR-H3 peptide neutralized HIV better than the linear form (Table 2 and Fig. 2). Syncytium inhibition was obtained with the two viral strains IIIB and SF2 (Fig. 3). Again the cyclic peptides

Table 1. Synthetic V3 and CDR peptides

Peptide(s)	Amino acid sequence
V3-IIIB	RKSIRIQRGPGRAFV
V3-MN	KRKCIHIGPGRAFYCTK
V3-RF	RKSITKGPGRVIYATG
V3-SF2	RKSIYIGPGRAFHTTG
CDR-L1	RASESVDDYGISFMH
CDR-L2	LLIYRASNLESGIPA
CDR-L3	YYCQQSNKDPLTFG
CDR-H1	GYTFTDHIMNWVKKR
CDR-H2	RIFPVSGETNYNQKFMG
CDR-H3	CDLIYYDYEEDYYFDY
CDR-H3/C1	CDLIYYDYEEDYYFDYC
CDR-H3/C2	CDLIYYDYEEDYYFDC
CDR-H3/C3	CDLIYYDYEEDYYFC
CDR-H3/C4	CDLIYYDYEEDYYC

Underlined sequences are CDRs of the mAb F58. Parts of framework regions (not underlined) were included. *, Disulfide bridges between native or introduced cysteines formed by chemical oxidation to create cyclic peptides.

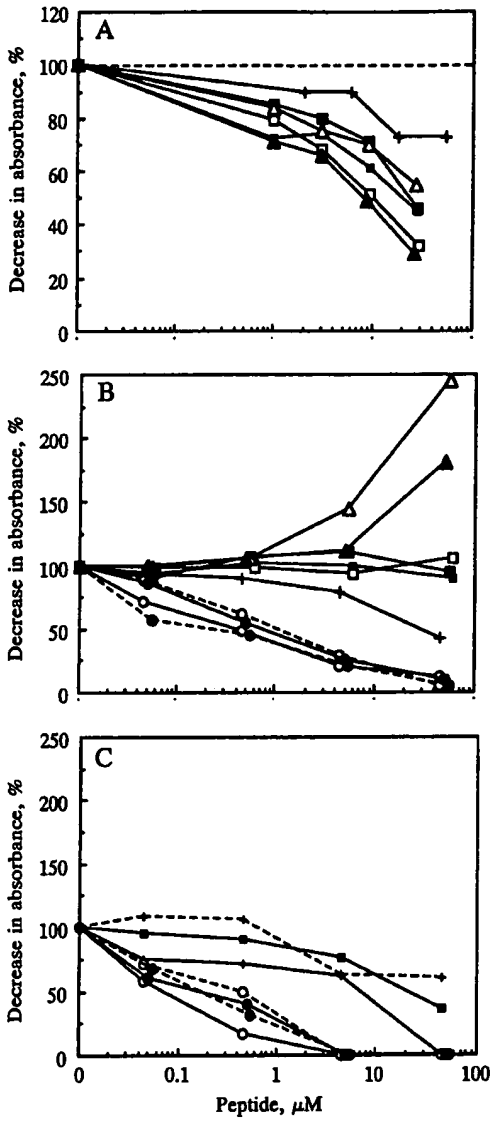

FIG. 1. (*A*) Blocking of the mouse mAb F58 from binding to the HIV-1 epitope peptide V3-IIIB by the CDR-H3 peptide and its peptide combinations. +, CDR-H3; ⊠, CDR-L1/H3; □, CDR-L2/H3; ■, CDR-L3/H3; △, CDR-H1/H3; ▲, CDR-H2/H3. Dotted line indicates mAb F58 binding. (*B*) Competition between mAb F58 and CDR peptides for binding to recombinant HIV-1 envelope protein rgp160. ⊡, CDR-L1; □, CDR-L2; ■, CDR-L3; △, CDR-H1; ▲, CDR-H2; +, CDR-H3; ○—○, CDR-H3/C1; ○----○, CDR-H3/C2; ●—●, CDR-H3/C3; ●----●, CDR-H3/C4. (*C*) Competition between mAb F58 and CDR peptides for binding to V3 peptides V3-MN, V3-RF, or V3-SF2. ■, CDR-H3 (V3-RF); +----+, CDR-H3 (V3-SF2). The following CDR peptides were tested against V3-MN: +—+, CDR-H3; ○—○, CDR-H3/C1; ○----○, CDR-H3/C2; ●—●, CDR-H3/C3; ●----●, CDR-H3/C4.

were more active than the linear CDR-H3 in reducing syncytium formation with both viral strains. The strongest syncytium-inhibiting effects were seen against the homologous IIIB viral strain but a clear inhibition of the heterologous SF2 strain was also seen (Fig. 3). The CDR-H1 peptide appeared to be toxic since no cells survived 1 h of peptide incubation together with 6 days of cultivation. The other CDR peptides did not affect the viability or proliferation of the cells.

DISCUSSION

We have identified a CDR sequence that specifically inhibits the HIV-1-neutralizing antibody from which it originates

Table 2. Neutralizing capacity of CDR peptides determined by p24 EIA

Peptide(s)	Neutralization* IIIB virus	SF2 virus
CDR-H3	0.60 (274)	>0.50 (>228)
CDR-H3/C1	0.38 (166)	0.43 (188)
CDR-H3/C2	0.30 (141)	0.38 (178)
CDR-H3/C3	0.35 (174)	0.78 (387)
CDR-H3/C4	0.40 (214)	0.70 (375)

*IC_{50}, mg/ml (μM).

from binding to its antigen. The results clearly indicate that the sequence CDLIYYDYEEDYYFDY in CDR-H3 is a major part of the discontinuous determinant involved in the antibody–antigen interaction. Mass spectroscopic analysis of the linear CDR-H3 peptide revealed that a fraction of the crude product consisted of the correct amino acid sequence. An interesting feature of this third hypervariable loop of the H chain is that it has a large number of tyrosines and negatively charged residues, whereas the V3 antigen predominantly has positively charged residues.

In native form as part of an antibody structure, CDRs form loops usually stabilized by β turns (20). After cyclic modifications, we improved the binding of CDR-H3 to V3 epitopes and virions compared to the linear CDR-H3. This is perhaps due to stabilization of a β turn not predominant in the linear peptide (21).

CDR-H3 interacted with V3 peptides of several HIV subtypes, thus inhibiting the reactivity of mAb F58 in blocking experiments. All combinations with CDR-H3 blocked mAb F58, the most competent being CDR-H2/H3. In competition experiments, single CDR-H3 had an advantage, perhaps because of its higher affinity or its smaller molecular size. An interesting finding was that CDR-H1, and to some extent CDR-H2 when alone, caused enhanced binding of mAb F58 to gp160. This may be taken to indicate an interaction of CDR-H1 with V3 outside of the GPGR sequence to enhance the GPGR availability to the mAb. Such a phenomenon indicates that single small peptides may be able to change the conformation of the protein to allow better binding to a definite site, in this case mAb F58 binding to GPGR. Another explanation may be that the CDR-H1 peptide has a general protein binding ability since it was toxic to our cell cultures.

The 14- to 17-amino acid residues in linear and cyclic forms representing CDR-H3 also neutralized HIV-1 *in vitro* and inhibited virus-induced syncytia. The concentration of linear CDR-H3 critical for neutralization appeared to be 0.6 mg/ml or 274 μM. It seemed that the peptide was more effective in

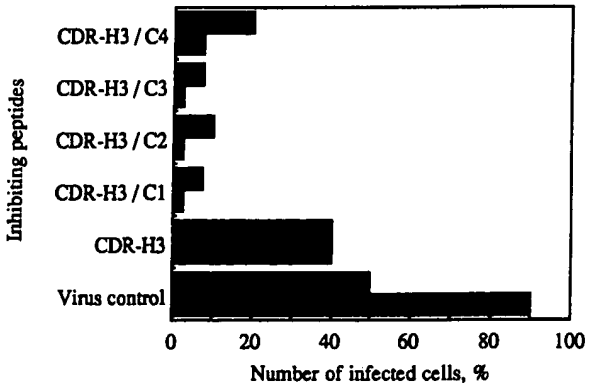

FIG. 2. Neutralizing capacity of 0.5 mg (220–270 μM) of CDR peptide per ml in C8166 cell cultures infected by HIV-1_{IIIB} or HIV-1_{SF2} determined by immunofluorescence. □, SF2 virus; ■, IIIB virus.

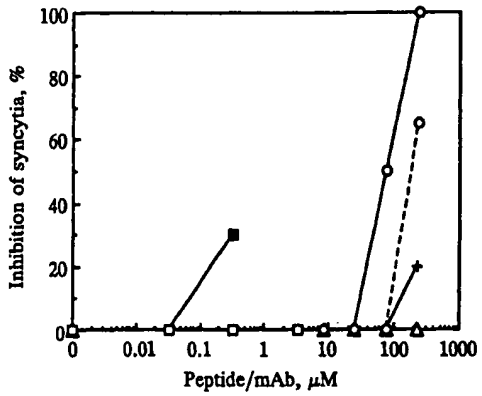

FIG. 3. Capacity of CDR-H3 and CDR-H3/C2 peptides and mAb F58 to inhibit syncytia of human C8166 T cells induced by HIV-1_{IIIB} and HIV-1_{SF2} determined by microscopy. All four cyclic CDR-H3 peptides had the same inhibiting capacity (data not shown). +, CDR-H3 inhibiting HIV-1 (IIIB); △, CDR-H3 (SF2); o—o, CDR-H3/C2 (IIIB); o----o, CDR-H3/C2 (SF2); ■, F58 (IIIB); □, F58 (SF2).

preventing syncytium induction by cell-to-cell spread of virus than by neutralizing free virions. During the fusion process that initiates syncytium formation between infected cells, the CDR binding to viral sequences may be more effective than on virions. Previously, mAb F58 has been shown to mediate type-specific neutralization in a concentration of 0.3–70 nM, depending on cell type and culture conditions. Cross-reactive neutralization required up to 3.3 μM mAb F58, depending on viral strain (4). Compared to our CDR peptides, the type-specific neutralization by whole F58 mAb thus occurs in a 10^2- to 10^3-fold lower concentration. It was obvious that modification of the CDR-H3 peptide by cyclization increased its binding capacity to antigen. We assume that this was due to a stabilization of the minimal recognition unit within this CDR that could not be further improved by shortening the cyclic peptide.

A biologically active peptide that inhibits reovirus 3 from attaching to its receptor was identified by Williams *et al.* (20). This peptide was derived from the second light-chain CDR of an anti-receptor mAb, while our active peptide is from CDR-H3 of mAb F58. Our results are not unexpected, though, since CDR3 appears to be the most variable of the CDRs in mouse V_H (9).

CD4 binding of virion gp120 is the first step in HIV-1 infection. The neutralization by antibodies has been suggested to be due to blocking of subsequent conformational changes, perhaps by blocking a protease cleavage site. Hattori *et al.* (6) suggested that cellular proteinases cleave the viral envelope glycoprotein, enabling the virus to enter the cell. A recombinant construction of the cellular proteinase tryptase TL_2 binds specifically to the envelope protein gp120. To elucidate whether our anti-V3 mAb F58 had any sequences in common with the cellular tryptase, a computer search for amino acid homology was performed with the CDR-H1 and -H3 regions of mAb F58. No homology was identified except in framework sequences.

Short peptides representing the V3 region itself have also been shown to inhibit HIV-1 infection *in vitro* (Vahlne, A., Horal, P., Hall, W., Rymo, L., Czerkinsky, C., Holmgren, J. & Svennerholm, B., Abstract, Annual Meeting of HIV and Retroviruses, National Cancer Institute, Washington, August 8–15, 1992).

The amino acids of the V3 region essential for V3-F58 binding have been described as I—GPGRA (22), common to many western HIV-1 strains (8). It may therefore be possible to delay progression to AIDS in HIV-1-infected individuals or even to prevent primary infection by CDR administration.

There should be several advantages of using CDR peptides instead of complete antibodies for passive immunization of HIV patients. The use of peptides would minimize secondary anti-idiotypic immune responses, higher molar concentrations could be given, and peptides would also penetrate better and to more compartments of the human body. One problem in passive immunization is the high possibility of secondary immune responses against the idiotopes of immunizing antibodies leading to rapid elimination of the antibody. As mentioned, such secondary responses may be reduced by immunizing only with the CDRs of the antibody. Anti-idiotypic antibodies (mouse anti-mAb F58) were studied to evaluate whether secondary immune responses could influence antibody–antigen interaction and consequently reduce mAb neutralizing capacity. Such inhibition of mAb F58 was seen in our mouse model, but the anti-idiotypes did not bind to any of the six linear CDRs. Their specificity may be directed to conformational CDR idiotopes. It is also possible that they bind to idiotopes located in the framework outside the hypervariable regions. The inhibition of mAb F58 interaction with antigen is then most probably due to steric hindrance of the antigen-interacting regions and not through direct binding to these regions.

Two major conclusions can be made: It is possible to inhibit the infectivity of HIV-1 by using a synthetic peptide corresponding to a H-chain CDR of a neutralizing antibody. This peptide appears to be cross-reactive since it inhibited the heterologous SF2 strain, differing in the V3 sequence from the IIIB strain to which mAb F58 was induced. The GPGR sequence is common between these strains and a broad neutralization of primary strains has previously been demonstrated (4). The results of this study reveal an additional field of application of synthetic peptides in the treatment of HIV-1 infection as biologically active miniantibodies.

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Recombinant Antibodies in Bioactive Peptide Design*

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Cristina Monfardini,[a,b] Thomas Kieber-Emmons,[c,d] Joan M. VonFeldt,[a,b,e] Brigid O'Malley,[a,b] Helga Rosenbaum,[a,b] A. Paul Godillot,[a,b] Kenneth Kaushansky,[f,h] Christopher B. Brown,[i] Donald Voet,[g] Daniel E. McCallus,[a,b] David B. Weiner,[a,b,c,h] and William V. Williams[a,b,f]

From the [a]Department of Medicine, Rheumatology Division, [b]Institute for Biotechnology and Advanced Molecular Medicine, and [c]Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine and [f]Childrens' Hospital of Philadelphia and the [g]Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104, the [f]Division of Hematology, University of Washington, Seattle, Washington 98195, and the [i]University of Calgary Health Sciences Center, Calgary, Alberta, Canada T2N 4N1

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is important in many immune and inflammatory processes. GM-CSF binds to specific cellular receptors which belong to a recently described supergene family. These receptors are potential targets for pharmacologic design, and such design depends on a molecular understanding of ligand-receptor interactions. One approach to dissecting out critical intermolecular interactions is to develop analogs of specific interaction sites of potential importance. Monoclonal antibodies have been employed for these purposes in prior studies. Here we present application of recombinant antibody technology to the development of analogs of a site on GM-CSF bound by a neutralizing anti-GM-CSF monoclonal antibody.

Polyclonal antisera with high titer neutralizing activity against human GM-CSF were developed in BALB/c mice. Purified immunoglobulins were prepared and used to immunize syngeneic mice. Anti-anti-GM-CSF was developed which demonstrated biological antagonist activity against GM-CSF-dependent cellular proliferation. RNA was extracted from spleen cells of mice with biologically active anti-anti-GM-CSF, cDNA synthesized, and polymerase chain reaction performed with primers specific for murine κ light chain V regions. Polymerase chain reaction products were cloned into the $pDAB_L$ vector and an expression library developed. This was screened with anti-GM-CSF neutralizing mAb 126.213, and several binding clones isolated. One clone (23.2) which inhibited 126.213 binding to GM-CSF was sequenced revealing a murine κ light chain of subgroup III. Comparison of the 23.2 sequence with the human GM-CSF sequence revealed only weak sequence similarity of specific complementarity determining regions (CDRs) with human GM-CSF. Structural analysis revealed potential mimicry of specific amino acids in the CDR I, CDR II and FR3 regions of 23.2 with residues on the B and C helices of GM-CSF. A synthetic peptide analog of the CDR I was bound by 126.213, specifically antagonized GM-CSF binding to cells and blocked GM-CSF bioactivity. These studies indicate the feasibility of using recombinant antibody libraries as sources of interaction site analogs.

Development of small molecular mimics of larger, polypeptide ligands is one approach to pharmacophore design. Several strategies are available for the development of such mimics, including the use of small oligopeptide analogs derived from native sequence (1–5), development of peptidic and non-peptidic analogs based on molecular structure data (6, 7), and analysis of alternative ligands (8). Alternative ligands that bind to the same site as the native ligand provide the opportunity to investigate structural and chemical constraints for binding in the setting of diverse backbone geometries. This has the potential to identify critical contact residues based on similar structural and chemical characteristics between the diverse ligands.

Prior studies have investigated a monoclonal antibody (mAb)[1], 87.92.6, which mimicked a neutralizing epitope on the reovirus type 3 hemagglutinin (9–12). 87.92.6 was bound both by a reovirus type 3 neutralizing mAb and the reovirus type 3 receptor. Sequence similarity between 87.92.6 light chain second complementarity determining region (CDR II) and the reovirus type 3 hemagglutinin (13) allowed the development of synthetic peptides and peptidomimetics which bound both the neutralizing mAb and the reovirus type 3 receptor. These peptides and peptidomimetics also demonstrated biological activity on reovirus type 3 receptor bearing cells. The use of anti-receptor mAbs as a source of sequence-structural information to aid in peptide design has allowed the development of similar biologically active peptides in several systems, including the platelet fibrinogen receptor (14), the thyroid-stimulating hormone receptor (15), and epitopes on the human immunodeficiency virus (16) and hepatitis B surface antigen (17).

Recombinant antibodies have been developed which are expressed in bacteria (18, 19) or on the surface of filamentous bacteriophage (20–23). The advantages of recombinant approaches to antibody development include the ability to rapidly screen thousands of clones simultaneously, the potential to detect binding moities poorly represented in the initial repertoire, and the potential to express isolated variable regions. While intact mAbs contain both light and heavy chain variable regions (V_L and V_H, respectively), recombinant antibodies can

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[1] The abbreviations used are: mAb, monoclonal antibodies; CDRs, complementarity determining regions; GM-CSF, granulocyte-macrophage colony-stimulating factor; PCR, polymerase chain reaction; IL, interleukin; cpm, counts/minute; IPTG, isopropyl-β-thio-galactopyranoside; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; rAb, recombinant antibody.

TABLE I
Oligonucleotide primers utilized for PCR and sequencing

Designation	Codons	Restriction site	Sequence[a]
3315	1 → 7	*Xba*I	CCCTCTAGAGACATTGTGCTGACCCAATCT
5591[b]	214 → 205	*Eco*RI	ACAGAATTCCCTGTTGAAGCTCTAGACAAT
931000[b]	114 → 110		CCAGTTGGTGCAGCATCA
pUC19 (3')			GTAAAACGACGGCCAGT
$pDAB_L$ (5')	−17 → −10		TTATTACTCGCTGCCCAACCAGCG

[a] Single underline delineates restriction sites, double underline delineates added stop codon at 207.
[b] Antisense oligonucleotide. Note that 5591 introduces a stop codon at codon 207.

be developed which express both V_L and V_H, or V_L or V_H alone. This limits the potential interaction sites of the recombinant antibody, allowing more precise delineation of critical interaction regions.

Here we describe the development of a recombinant light chain library in *Escherichia coli* derived from mice immunized with polyclonal anti-GM-CSF. This library was screened with a previously described neutralizing anti-GM-CSF mAb 126.213 (24) which inhibits GM-CSF binding to HL-60 cells, neutralizes GM-CSF induced colony formation, and competes with the α chain of the GM-CSF receptor for GM-CSF binding (25). Screening with radioiodinated 126.213 yielded several binding clones, including one that inhibited immunoprecipitation of GM-CSF by 126.213. Comparison of the recombinant V_L sequence with the human GM-CSF sequence revealed only weak similarity with GM-CSF, but structural analysis suggested mimicry of residues on the B and C helices of GM-CSF by a site chiefly made up of the CDR I region of 23.2. A synthetic peptide corresponding to the CDR I was bound by the neutralizing anti-GM-CSF mAb and specifically inhibited GM-CSF binding and the growth of GM-CSF-dependent cells. These studies suggest a structural basis for recombinant antibody mimicry of a predominately helical molecule (human GM-CSF), demonstrate a bioactive peptide analog of a GM-CSF site implicated in receptor binding, and indicate the feasibility of using recombinant antibody libraries as sources of interaction site analogs.

MATERIALS AND METHODS

Bacterial Strains—E. coli DH5α competent cells (Life Technologies, Inc.) were used for transformation. Bacteria were grown in Luria broth containing 100 mg/ml ampicillin (LB/amp) (26, 27). Solid media contained 1.5% agar (Difco Inc.).

Enzymes and Oligonucleotides—Restriction endonucleases and T4 DNA ligase were purchased from Life Technologies, Inc. Enzyme reaction conditions were according to those of the supplier. Oligonucleotides for PCR primers and for Southern blotting were synthesized by the DNA Synthesis Facility of the Wistar Institute. The primers were selected by analysis of immunoglobulin sequences as published by Kabat *et al.* (28). The specific primers are listed in Table I. PCR amplification employed primers 3315 (relatively specific for the murine Vκ III family) and 5591 (near the 3' end of the Cκ coding region). Note that primer 5591 introduces a stop codon at codon 207, resulting in a truncated light chain lacking the carboxyl-terminal 8 amino acids including the cysteine at position 214. This should result in production of light chains which are predicted to remain monomers. Primers were tested on various hybridoma cell lines in the laboratory prior to their use in library construction. In these studies amplified sequences were isolated from the gels cloned and sequenced to verify the utility of the primers and their specificity for amplification of Ig variable regions (data not shown).

Cell Lines and Proliferation Assay—CTLL cells and the proliferation assay in response to IL-2 or rat spleen concanavalin A was described previously (29). AML 193 cells were obtained from the American Type Culture Collection (ATCC), and MO7E cells were from R. Zollner, Genetics Institute (Cambridge MA). AML 193 was grown serum free in Iscove's modified Dulbecco's medium with insulin (10 μg/ml), transferrin (5–10 units/ml), 1% OPI media additive (oxalate, pyruvate, and insulin), and GM-CSF (0.5 ng/ml). MO7E was grown in RPMI 1640 with 10% heat-inactivated fetal calf serum, Pen/strep, L-glutamine, and GM-CSF 0.5 ng/ml. For proliferation assays, 2×10^4 AML 193 or MO7E cells were cultured per well in 96-well round bottomed plates in the above medium along with test antisera in a final volume of 200 μl. Following a 3–5 day incubation, tritiated thymidine (1 μCi/well) was added for an additional 18 h, the cells harvested onto glass fiber filters utilizing a PhD cell harvester, and counts/minute incorporated determined in a standard liquid scintillation system.

Development of Anti-GM-CSF and Anti-anti-GM-CSF—Recombinant human GM-CSF (obtained from Bachem Biosciences, Philadelphia, PA) was used to immunize BALB/c mice as described previously (30). Serum was obtained 1 week following each boost with antigen. Antisera from three to five animals were pooled for the assays performed. Following the third boost, significant neutralizing titers against human GM-CSF-dependent cellular proliferation were demonstrated (Fig. 1). The mice were bled after five boosts and IgG purified from serum by affinity chromatography with Sepharose-protein A. This was used to immunize syngeneic BALB/c mice (50 μg of purified IgG/immunization) and serum obtained following each boost. The sera were assayed for inhibition of GM-CSF-dependent proliferation (see below), and significant (>50%) inhibition was seen following the eighth boost against both MO7E and AML193 cells (Fig. 1 and data not shown). Mice that exhibited neutralizing activity on this assay served as spleen cell donors.

Amplification of Anti-anti-GM-CSF Immunoglobulin Light Chain Variable Regions (V_L)—Spleenocytes were isolated from four anti-GM-CSF immunized mice who displayed neutralizing activity against GM-CSF-dependent proliferation. A cell suspension was prepared and lymphocytes isolated by Ficoll-Hypaque density gradient centrifugation. RNA was extracted with the RNAzol kit (Biotecx Laboratories Inc., Houston, TX), according to the manufacturer's instructions. Following isolation, the RNA was precipitated with isopropyl alcohol, pellets washed in 70% ethanol, and rotary evaporated. The dried pellets were resuspended in 50 μl of diethylpyrocarbonate-water and RNA quantified spectrophotometrically.

For reverse transcription, 10–20 μg of RNA in 10 μl was utilized to synthesize cDNA primed with random hexamers in the following reaction mixture: 3 μl of Maloney murine leukemia virus reverse transcriptase with 6 μl of 5 × reverse transcriptase buffer, 1.5 μl of RNase inhibitor, and 3 μl of 0.1 M dithiothreitol (all from Life Technologies, Inc.), 3 μl (100 pmol) of random hexamers (from Pharmacia LKB Biotechnol), and 1 μl of 40 mM dNTPs (10 mM in each dNTP, from Boehringer Mannheim, GmbH W., Germany). Following a 10-min preincubation at 25 °C, the reaction was carried out for 1 h at 42 °C, then 95 °C for 5 min followed by storage at −20 °C until use.

For PCR amplification, the oligonucleotide primers 3315 and 5591 listed in Table I were employed at 0.5 nM/ml final concentrations. The relative position of these primers on Igκ cDNA is shown in Fig. 2. The PCR mixture (100 μl) consisted of 10 μl of PCR primers, 16 μl of dNTPs (final concentration 200 μM in each dNTP), 10 μl of PCR buffer (10 ×; Perkin-Elmer Cetus), 61.5 μl of dH_2O, 2 μl cDNA, and 1.2 units of *Taq* polymerase (Perkin-Elmer Cetus). Amplification was carried out in a Programmable Thermal Cycler (MJ Research, Watertown, MA). The amplification program was 94 °C for 3 min followed by five cycles of 94 °C for 60 s, 52 °C for 60 s, 72 °C for 60 s; followed by 25 cycles of 94 °C for 60 s, 52 °C for 90 s, and 72 °C for 120 s. Following 30 cycles, the temperature was held at 72 °C for 7 min. Positive amplification was determined by agarose gel electrophoresis. The PCR products were cloned into the $pDAB_L$ plasmid, which is of utility for protein expression as has been published previously (31, 32). PCR products and plasmid DNA were cut with the appropriate endonucleases and plasmid DNA

was treated with calf intestinal phosphatase (Boehringer Mannheim), followed by ligation using 1 unit of T4 DNA ligase overnight at 16 °C. Ligation mixtures were transformed into *E. coli* DH5α competent cells as described by the manufacturer.

Library Characterization—Competent *Epicurian coli* cells (the Cell Center, University of Pennsylvania) transformed with the amplified Vκ/pDAB$_L$ library was plated on LB/amp plates. Control ligated pDAB$_L$ vector produced four to five colonies/plate, while the appropriately ligated Vκ/pDAB$_L$ transformants produced 175–465 colonies/plate. Inserts were confirmed by plasmid miniprep analysis (26, 27), which revealed appropriately sized inserts in 75% of colonies. Approximately 2,000 colonies on 10 plates were screened in this study.

Protein Expression—Bacterial clones possessing the Vκ genes inserted into pDAB$_L$ were plated onto LB/amp plates. Control plates contained *E. coli* transformed with either pDAB$_L$ alone, pUC19,or pUC18. Following overnight growth, replica plating, and additional overnight growth, 0.45-m nitrocellulose filters were placed on the bacterial plate. Filters were lifted to other LB/amp plates on which 50 μl of isopropyl-β-thio-galactopyranoside (IPTG) (25 mg/ml; Stratagene, La Jolla, CA) had been spread and were then incubated for 4 h at 37 °C. ilters were then exposed to chloroform vapor for 15 min and incubated overnight (with shaking) in lysis buffer (100 mM Tris-Cl, pH 7.8,150 mM NaCl,5 mM $MgCl_2$,1.5% bovine serum albumin (BSA),1 μg/ml pancreatic DNase I,and 40 μg/ml lysozyme). Filters were then blocked for 4 h with blocking buffer (5% non-fat dry milk and 0.05% BSA in phosphate-buffered saline (PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH .4)). Following blocking, filters were screened for specific variable region expression as noted below.

For some experiments, lysates were prepared of bacteria expressing the recombinant antibody fragments. Lysates of *E. coli* XL1 Blue cells (Stratagene, La Jolla, CA) were prepared either from unmanipulated bacteria or *E. coli* transformed with pDAB$_L$ alone, or the various V_L regions ligated into pDAB$_L$. Colonies were grown overnight in LB/Amp, and 500 μl used to seed 5-l cultures grown to ~0.6 A_{450} units in Superbroth (Cell Center, University of Pennsylvania), then induced with 1 mM IPTG for for 4–12 h. The cells were centrifuged (10,000 revolutions/min for 30 min) and the pellets dissolved in 2 ml of lysis buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and aprotinin diluted 1:100 from a concentration of 2.1 mg/ml, all from Sigma). These cells were sonicated for 45 s on ice and clarified by centrifugation (11,000 × *g* for 15 min at 4 °C) and the supernatant (lysate) used as sources of V_L fragments.

Library Screening—For binding of ^{125}I-labeled 126.213 or control mAb ID6 (33) blocked filters were incubated for 2 h at 37 °C with ^{125}I-labeled mAb (purified by staphylococcal protein A affinity chromatography), 500,000–1,000,000 cpm/ml, labeled by the chloramine T method (9) in PBS containing 1% BSA and 0.05% Tween-20 (PBS-BSA). Filters were washed extensively with PBS-BSA and autoradiographed (Kodak XRP film) for 2–24 h.

DNA Sequencing—Double-stranded DNA sequencing employed the primers listed in Table I and followed previously published protocols (34). Sequencing proceeded in both the 5′ and 3′ orientations to confirm all sequence information.

Western Blotting—Bacterial lysates (prepared as above) or recombinant human GM-CSF were run on 15% SDS-polyacrylamide gel in reducing sample buffer (2% SDS, 50 mM Tris-HCl, pH 6.8, 10% glycerol, 0.001% bromphenol blue) with 500 μg of bacterial protein (as determined by the Bio-Rad protein assay) loaded in each well. Following electrophoresis, the gel was transferred to Immobilon P transfer membranes (Millipore) as described (35), and the blot blocked with 1% bovine serum albumin, 5% non-fat dry milk, 0.05% Tween 20 in PBS (blocking buffer) for >1 h at 37 °C or overnight at 4 °C. The blots were then incubated with 5 ml of purified mAb 126.213 diluted to 2 μg/ml in blocking buffer for 2 h at 37 °C, and washed four times in PBS, 0.05% Tween 20 (wash buffer). Polyclonal goat anti-mouse IgG (Sigma) (radioiodinated as described (9)) was then added (1,000 cpm/μl in blocking buffer) and the blot incubated for 2 h at 37 °C, washed extensively in wash buffer, dried, and exposed for 12–72 h to Kodak XAR film.

Immunoprecipitation—5 μg of purified 126.213 was reacted with protein G beads (Sigma) in Eppendorf tubes and rotated overnight at 4 °C. The tubes were centrifuged and the liquid aspirated. The beads were then washed three time with lysis buffer (1% Triton, 0.05% SDS, 10 mM Na_2HPO_4-NaH_2PO_4, 150 mM NaCl, 5 mM EDTA, 100 μM Na_3VO_4, and 5 μg/ml aprotinin all from Sigma) to remove unbound antibody. The beads were resuspended in 100 μl of lysis buffer, and ^{125}I-GM-CSF was added to the tubes in the presence or absence of inhibitors (100 μl of total volume) and rotated at 4 °C for 1 h. The tubes were then centrifuged, the liquid discarded, and the beads washed three times. The beads were then resuspended in 2 × sample buffer (0.5 M Tris-HCl, pH 6.8, 16% glycerol, 3.2% SDS, 8% 2-mercaptoethanol, and 0.04% bromphenol blue (all from Sigma) in distilled H_2O) and heated at 95 °C for 5 min to dissociate bonds. Samples were then loaded onto 10% SDS-polyacrylamide gel electrophoresis gels and analyzed by autoradiography as described (35, 36).

Radioreceptor Binding Assay—This was modified from previously published protocols (37, 38). Briefly, HL-60 cells (from ATCC) were grown in RPMI 1640 with 10% fetal calf serum and added L-glutamine. 1–2 × 10^6 HL-60 cells were washed twice in RPMI 1640 with 1% BSA and 25 mM HEPES, pH 7.4 (binding buffer), centrifuged, and incubated with inhibitors as noted in figure legends in a 25-μl volume for 1 h at room temperature. 0.5 nM of ^{125}I-GM-CSF (118 μCi/μg, from DuPont NEN) was then added for 30 min at room temperature, the cells layered over 500 μl of chilled fetal calf serum, centrifuged, and the pellets counted.

Peptide Synthesis—All peptides were synthesized by solid-phase methods as described previously (9–12) by the Wistar Institute Peptide Synthesis Facility or Macromolecular Resources at Colorado State University, deprotected, and released from the resin using anhydrous HF.

Enzyme-linked Immunosorbent Assay (ELISA)—ELISA was performed with polystyrene plates (Dynatech Laboratories Inc.) coated with peptide by evaporation of peptides (at the concentrations noted) in distilled water overnight at 37 °C. The wells were washed with PBS, blocked with 0.05% Tween, 2% BSA in PBS, and washed with PBS. Primary antibodies were added at varying dilutions for >1 h at 37 °C. After washing, secondary antibody, goat anti-mouse conjugated to horseradish peroxidase (Sigma) was added per well in 1% BSA in PBS for 1–2 h at 37 °C. The substrate used for color development was 3,3′,5,5′-tetramethyl-benzidine dihydrochloride (Sigma). The wells were decanted, washed extensively, and absorbance of samples was measured in a plate reader (MR 5000; Dynatech Laboratories) and expressed as A_{450} nm. Specific values were determined by subtracting the absorbance measured from uncoated wells from the absorbance to peptide coated wells (39).

RESULTS

Construction and Screening of Anti-anti-GM-CSFV $_LC_\kappa$ Library—Polyclonal neutralizing antibodies against human GM-CSF raised in BALB/c mice were used to develop syngeneic anti-anti-GM-CSF with neutralizing activity (Fig. 1). The PCR was used to amplify immunoglobulin Vκ genes from these mice. Oligonucleotide primers for amplification of immunoglobulin genes were chosen based on conserved DNA sequences found in V_L variable framework regions and from the κ constant region domain. The 5′ primer used in these experiments was relatively specific for the murine VκIII family. The 3′ primer introduced a stop codon at position 207, eliminating 8 amino acids including the carboxyl-terminal cysteine residue, thereby eliminating the tendency for the produced light chains to dimerize. Spleen cells were isolated, RNA extracted, and cDNA synthesized. This served as a template for PCR amplification of the V_LC_κ regions. Bands of the expected size (~680 base pairs) were observed following agarose gel electrophoresis. This amplification was specific as control cellular DNA from human T lymphocyte cell lines did not yield a PCR product (data not shown).

The PCR products were ethanol precipitated (to remove residual primer DNA) and digested with appropriate restriction endonucleases (*Xba*I and *Eco*RI). These were ligated into similarly restricted, alkaline phosphatase-treated pDAB$_L$. Following ligation, the reaction products were transformed into *E. coli* DH5α cells and plated onto 30 LB/amp plates. This V_LC_κ library was then screened after induction with IPTG with radioiodinated neutralizing mAb 126.213, which specifically neutralizes GM-CSF activity. Thirty filters containing 500–1,000 colonies each were screened in this manner. A representative filter is shown in Fig. 2*A*. Based on the observed binding of ^{125}I-126.213 to colonies we picked 30 reactive colonies. These were expanded and replated and rescreened using fresh ^{125}I-126.213 and a control mAb (ID6) specific for HIV-1 gp120 (33). Approximately 50% of the filters were bound by ^{125}I-126.213

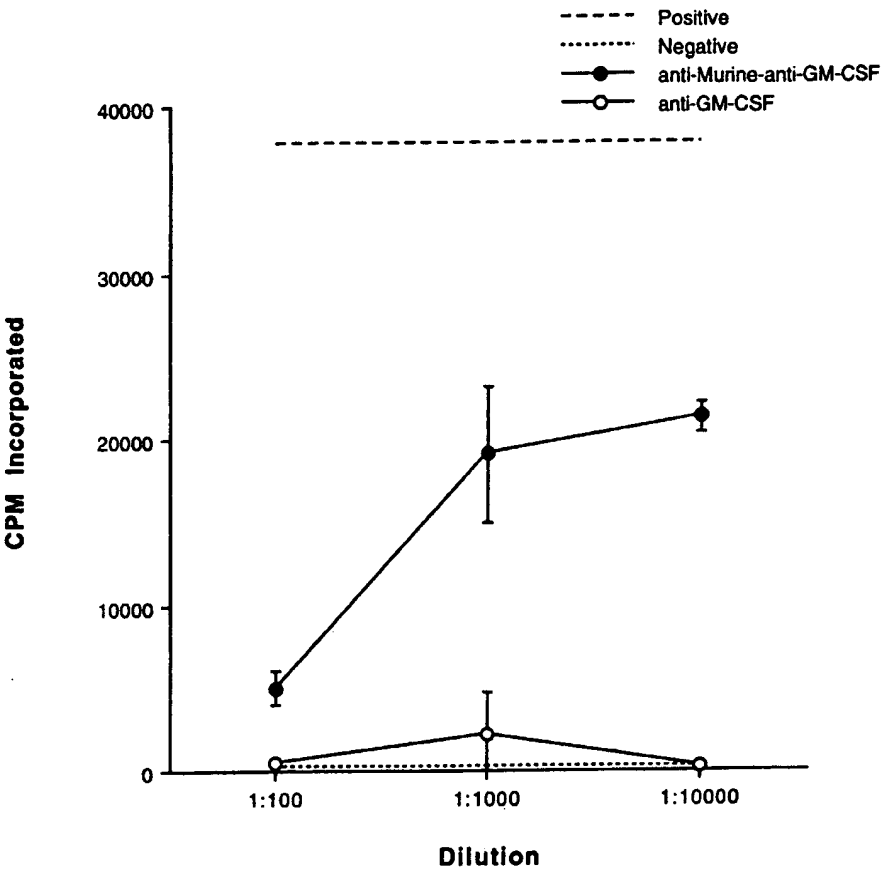

FIG. 1. **Biological activity of antisera.** Proliferation of the human GM-CSF-dependent cell line MO7E was performed as noted under "Materials and Methods" in the presence of varying dilutions of murine anti-GM-CSF (following the fifth boost) and murine anti-anti-GM-CSF (following the ninth boost). Counts/min incorporated ± the standard deviation of triplicate wells is shown for various dilutions of antisera. In similar experiments, the inhibition induced by anti-GM-CSF titered out at 1:20,000 to-: 100,000 dilutions.

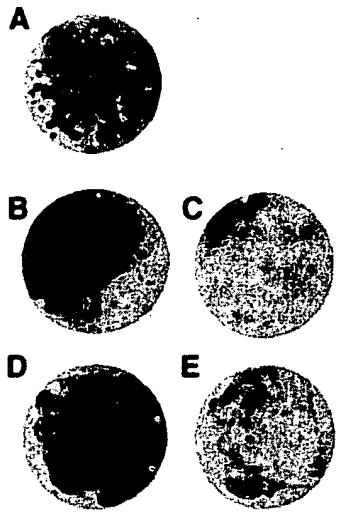

FIG. 2. **Library screening.** *A*, first round library screening was carried out on 30 filters lifted from 30 LB/amp plates representing a total of ~15,000–20,000 colonies. A representative filter is shown here. *B*, second round screening of one positive clone (clone 23.2) replated and probed with ^{125}I-126.213. Compare with *panel C*, second round screening of a control clone with an irrelevant V_L region. *D*, third round screening of clone 23.2. Compare with *panel E*, *E. coli* transformed with $pDAB_L$ alone.

but not by ^{125}I-ID6 following the second round of screening (see Fig. 2, *B* and *C*, for representative filters screened with ^{125}I-126.213). Most of these were bound by ^{125}I-126.213 in subsequent rounds of screening (Fig. 2, *D* and *E*). Ten colonies which were consistently bound by ^{125}I-126.213 but not ^{125}I-ID6 in subsequent assays were selected for further characterization.

Characterization of V_LC_κ Regions—Western blot analysis was performed of bacterial lysates prepared from the bacterial colonies consistently bound by 126.213. For Western analysis, lysates were prepared from *E. coli* transformed with the $pDAB_L$ plasmid alone, or containing the specific light chain inserts. The cultures were then either left uninduced or induced with IPTG, bacterial lysates prepared, separated electrophoretically, and transferred to Immobilon filters. These were probed with 126.213 followed by ^{125}I-goat anti-mouse IgG and analyzed by autoradiography. A typical Western blot is shown in Fig. 3*A*. This compares cultures of bacteria both uninduced and following induction with IPTG. As can be seen, IPTG induces the appearance of a ~21–23 kDa band for the clones containing specific V_L regions, while only nonspecific bands are present in the cultures transformed with $pDAB_L$ alone. Notably, this gel was run under non-reducing conditions, suggesting that the light chains do not dimerize, instead remaining as monomers. The molecular mass of the band detected is somewhat lower than the 23 kDa predicted for the isolated light chain. This may be due to inaccuracy of the molecular weight markers used or could reflect compact folding of the V_LC_κ fragments.

The neutralizing mAb 126.213 specifically immunoprecipitates ^{125}I-GM-CSF. This assay allowed investigation of the ability of various rAb V_LC_κ regions to compete with ^{125}I-GM-CSF binding to 126.213. Of the 10 rAb V_LC_κ regions screened, only one (clone 23.2) reproducibly inhibited immunoprecipitation by 126.213 (Fig. 3*B*). Inhibition with the lysates from bacteria transformed with 23.2 reproducibly inhibited immunoprecipitation on multiple experiments (Fig. 3*B* and data not shown). Inhibition was much greater for IPTG-induced cell lysates compared with uninduced lysates. Clone 23.2 was se-

lected for further characterization.

Inhibition of GM-CSF Binding to HL-60 Cells by 23.2—GM-CSF specifically binds GM-CSF receptors present on HL60 (human myelomonocytic leukemia) cells, and this binding is inhibited by 126.213 (24). We examined the ability of rAb 23.2 to inhibit binding of ^{125}I-GM-CSF to HL60 cells on a standard cellular binding assay. In this assay, HL-60 cells were preincubated with lysates from *E. coli* induced with IPTG following transformation with the 23.2 plasmid or an irrelevant plasmid (pUC18). The counts/minute bound are shown *versus* increasing amounts of lysate added in Fig. 4. 23.2 transformed bacterial lysates inhibited binding of ^{125}I-GM-CSF to HL-60 cells, while control lysates had no effect. This result indicates that 23.2 competes with GM-CSF for binding to a site on HL-60 cells and may bind to the GM-CSF receptor present on these cells.

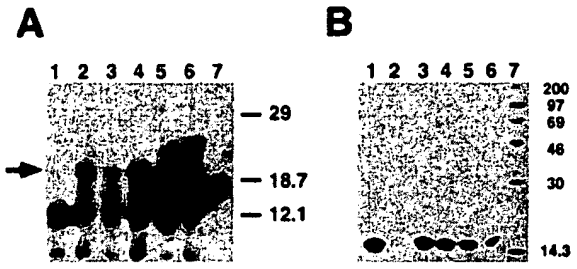

FIG. 3. **Characterization of rAb V_L regions.** *A*, Western blot analysis of rAb fragments. *E. coli* transformed with various plasmids were induced or left uninduced, lysates prepared, and Western blotting performed with 126.213 as the primary antibody as noted under "Materials and Methods." *Lanes* were as follows: *1*, clone 23.2 uninduced; *2*, clone 23.2 induced; *3*, clone 5.1 uninduced; *4*, clone 5.1 induced; *5*, $pDAB_L$ alone uninduced; *6*, $pDAB_L$ alone induced; *7*, 300 ng of GM-CSF (positive control). Molecular weight markers are indicated. The *arrow* indicates the band specifically induced. *B*, inhibition of immunoprecipitation by 23.2. Immunoprecipitation of ^{125}I-GM-CSF was performed as noted under "Materials and Methods." Lysates of *E. coli* expressing 23.2 or control (irrelevant clone) were prepared, protein quantified, and 400 μg used to inhibit immunoprecipitation. Inhibitors were added as follows: *1*, $pDAB_L$ alone induced; *2*, 300 ng of GM-CSF; *3*, clone 25.1 uninduced; *4*, clone 25.1 induced; *5*, clone 23.2 uninduced; *6*, clone 23.2 induced; *7*, ^{14}C molecular weight markers.

Sequence of Clone 23.2—As clone 23.2 was specifically bound by mAb 126.213, and competed with GM-CSF for binding to 126.213 and to HL-60 cells, the 23.2 insert was sequenced. The nucleic acid sequence and derived amino acid sequence of 23.2 is shown in Fig. 5. The 23.2 V_L region is a member of the murine Vκ III family as defined by Kabat *et al.* (28), or the Vκ 21 group as defined by Weigert *et al.* (40), with the J region derived from the Jκ1 family (40). Data base searching reveals that the 23.2 V/J amino acid sequence is very similar to the previously described Vκ 21 hybridoma light chains 6684 and 7940 derived from NZB mice (40), differing by only 6 amino acid substitutions from 6684 and 8 substitutions from 7940.

The intact 23.2 sequence and the individual CDR sequences were compared with the human GM-CSF sequence using the Bestfit, Gap, Wordsearch, and Segments programs of the Wisconsin package (41). Several regions of weak sequence similarity were noted which involved CDR regions of 23.2. Prior studies of 126.213 used murine/human chimeric forms of GM-CSF to map interaction sites (24). These studies suggested that residues 77–83 were critical for 126.213 binding to GM-CSF. We noted weak homology of the CDR I and CDR II with this epitope. An additional region of weak sequence similarity was also seen between amino acids 54–61 of GM-CSF and the CDR III of 23.2. Interestingly, amino acids 54–61 (on the B helix of GM-CSF) lie immediately adjacent to amino acids 77–83 (on the C helix) in the crystal structure of GM-CSF (42). However, the weak sequence similarity seen here indicated that the mimicry of GM-CSF by 23.2 might be better accounted for on a structural level.

Structural Analysis of GM-CSF Mimicry by 23.2—Structural analysis of 23.2 was carried out following development of mo-

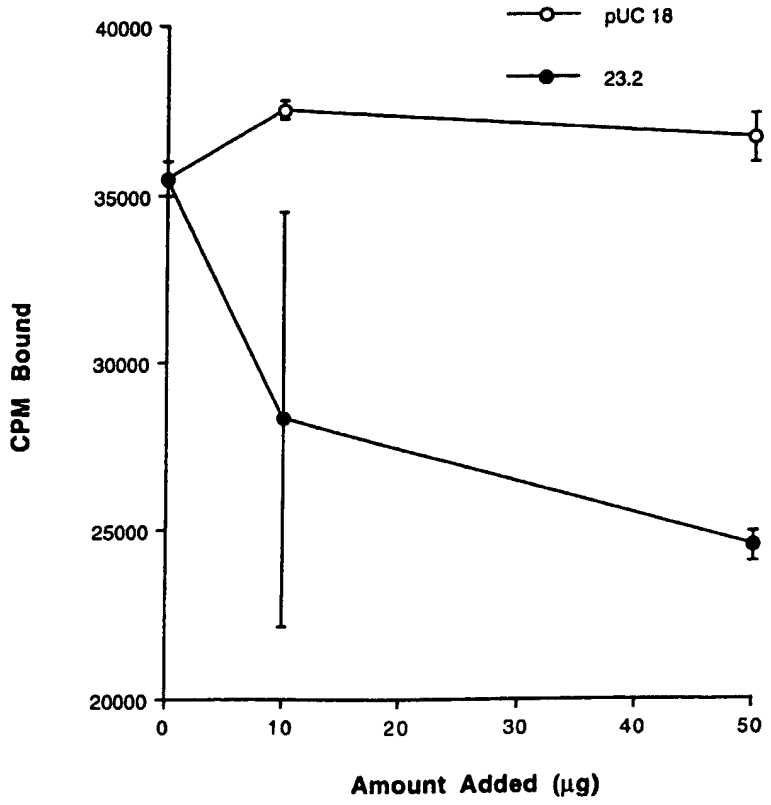

FIG. 4. **Inhibition of ^{125}I-GM-CSF binding to HL-60 cells by 23.2.** The binding assay was performed as noted under "Materials and Methods" using 2 × 10^6 HL-60 cells, in the presence or absence of increasing amounts of 23.2 or control (pUC18) lysates. The counts/min (*cpm*) bound ± standard error of replicate determinations for two lysate preparations are shown.

NUCLEIC ACID AND DEDUCED AMINO ACID SEQUENCE OF CLONE 23.2

```
1/1                                                 31/11
GAC ATT GTG CTG ACC CAA TCT CCT GCT TCC TTA ACT GTA TCT CTG GGG CAG AGG GCC ACC
ASP ILE VAL LEU THR GLN SER PRO ALA SER LEU THR VAL SER LEU GLY GLN ARG ALA THR
FR 1---------------------------------------------------------------------------->

61/21                                               91/31
ATC TCA TGC AGG GCC AGC AAA AGT GTC AGT TCA TCT GGC TAC AGT TAT ATG CAC TGG TAC
ILE SER CYS ARG ALA SER LYS SER VAL SER SER SER GLY TYR SER TYR MET HIS TRP TYR
----------> CDR I------------------------------------------------------> FR 2-->

121/41                                              151/51
CAA CAG AAA CCA GGG CAG CCA CCC AAA GTC CTC ATC TAT CTT GCA TCC AAC CTA GAA TCT
GLN GLN LYS PRO GLY GLN PRO PRO LYS VAL LEU ILE TYR LEU ALA SER ASN LEU GLU SER
-------------------------------------------------> CDR II-------------------->

181/61                                              211/71
GGG GTC CCT CCC AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC TTC ACC CTC AAC ATT CAT
GLY VAL PRO PRO ARG PHE SER GLY SER GLY SER GLY THR ASP PHE THR LEU ASN ILE HIS
FR 3---------------------------------------------------------------------------->

241/81                                              271/91
CCT GTG GAG GAG GAG GAC GCT GCA ACC TAT TAC TGT CAG CAC AGT AGG GAG CTT CCG TGG
PRO VAL GLU GLU GLU ASP ALA ALA THR TYR TYR CYS GLN HIS SER ARG GLU LEU PRO TRP
-----------------------------------------------> CDR III---------------------->

301/101                                             331/111
ACG TTC GGT GGA GGC ACC AGG CTG GAA ATC AAA CGG GCT GAT GCT GCA CCA ACT GTA TCC
THR PHE GLY GLY GLY THR ARG LEU GLU ILE LYS ARG ALA ASP ALA ALA PRO THR VAL SER
--> FR4------------------------------------> C kappa--------------------------->

361/121                                             391/131
ATC TTC CCA CCA TCC AGT GAG CAG TTA ACA TCT GGA GGT GCC TCA GTC GTG TGC TTC TTG
ILE PHE PRO PRO SER SER GLU GLN LEU THR SER GLY GLY ALA SER VAL VAL CYS PHE LEU
---------------------------------------------------------------------------->

421/141                                             451/151
AAC AAC TTC TAC CCC AAA GAC ATC AAT GTC AAG TGG AAG ATT GAT GGC AGT GAA CGA CAA
ASN ASN PHE TYR PRO LYS ASP ILE ASN VAL LYS TRP LYS ILE ASP GLY SER GLU ARG GLN
---------------------------------------------------------------------------->

481/161                                             511/171
AAT GGC GTC CTG AAC AGT TGG ACT GAT CAG GAC AGC AAA GAC AGC ACC TAC AGC ATG AGC
ASN GLY VAL LEU ASN SER TRP THR ASP GLN ASP SER LYS ASP SER THR TYR SER MET SER
---------------------------------------------------------------------------->

541/181                                             571/191
AGC ACC CTC ACG TTG ACC AAG GAC GAG TAT GAA CGA CAT AAC AGC TAT ACC TGT GAG GCC
SER THR LEU THR LEU THR LYS ASP GLU TYR GLU ARG HIS ASN SER TYR THR CYS GLU ALA
---------------------------------------------------------------------------->

601/201                                             631/211
ACT CAC AAG ACA TCA ACT TCA CCC ATT GTC TAG AGC TTC AA
THR HIS LYS THR SER THR SER PRO ILE VAL AMB
------------------------------> PCR Primer------------------------>
```

FIG. 5. **Nucleic acid and derived amino acid sequences of clone 23.2.** Sequencing was performed by double-stranded DNA sequencing with *Taq* polymerase, as described previously, using both the PCR primers and primers derived from the $pDAB_L$ plasmid. *FR*, framework; codon numbering (above the sequence) is according to Kabat *et al.* (28) with codon one corresponding to the first amino acid residue of the FR1 region. Leader peptide sequence is not shown.

lecular models of the V_L domain. A molecular model of the light chain was developed by examining sequence homologies of the 23.2 sequence with sequences of crystallographically known light chain structures. Identification of crystallographic templates for the light chain model included examining the length of the respective CDRs to match those of the template. As many light chain structures display equivalent CDR II and CDR III lengths, several alternative models were developed. Model 1 was developed using as a template the antibody 50.1 (Iggb), an antibody directed against the V3 loop of HIV gp120. This template displayed equivalent CDR lengths with the 23.2 sequence. The CDRs and framework (FR) regions of the 50.1 template were mutated to those of 23.2 using the program Insight (Biosym Technologies). The side chain angles of the

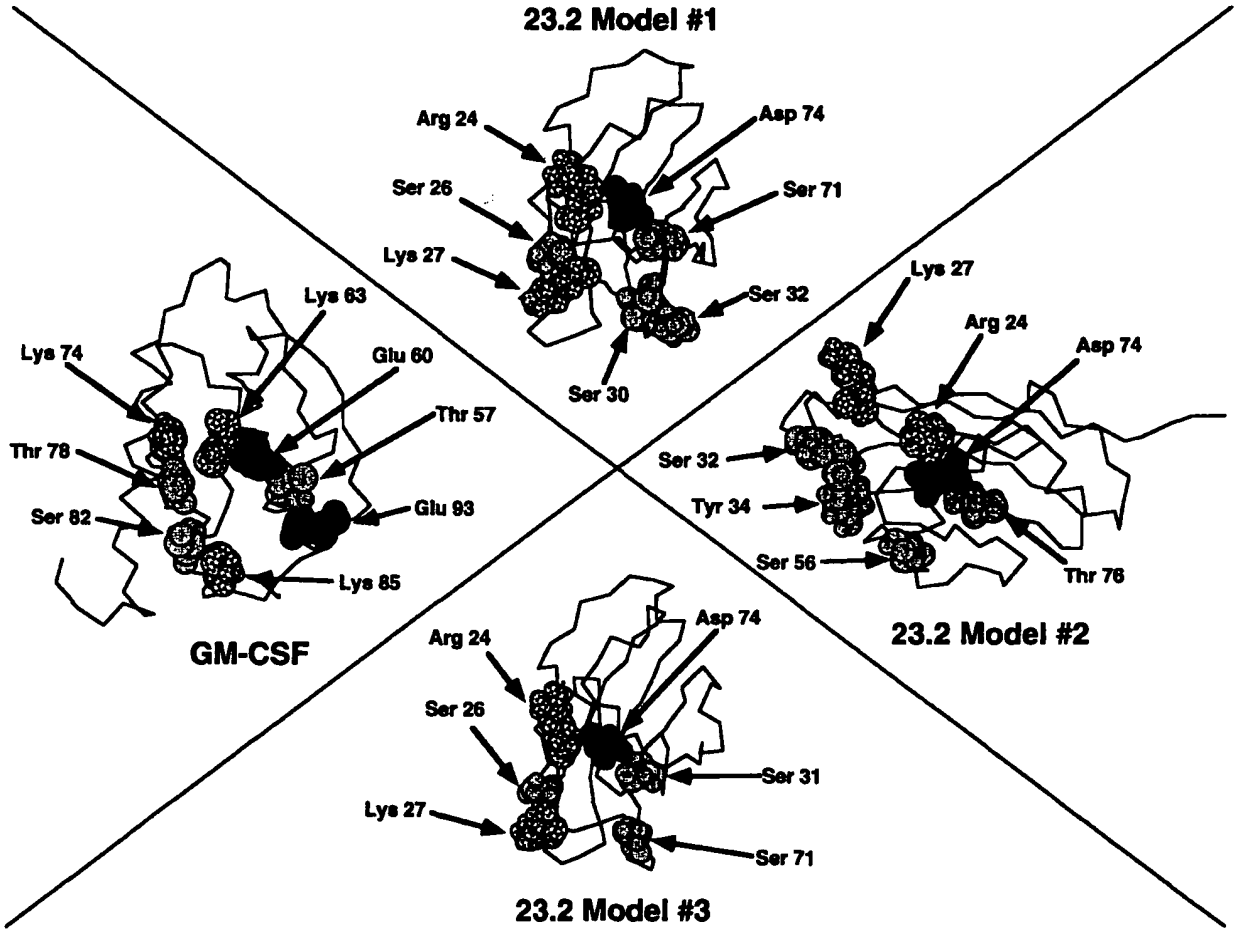

FIG. 6. **Structural basis for mimicry.** The structure of GM-CSF was determined from coordinates derived from the crystal structure (J. M. LaLonde, K. Swaminathan, and D. Voet, manuscript in preparation), displayed on the MacImdad program (Molecular Applications Group, Palo Alto, CA) on a Macintosh Quadra 950 computer. The 23.2 V_L models were derived as described under "Results." The GM-CSF view is directed at the B and C helices, while the 23.2 models' view is directed at the CDR I region. Specific residues implicated in mimicry are indicated. The models are further discussed in the text.

substituted residues were set according to angles identified in a data base of side chains. Each CDR and FR region were changed individually, followed by 1000 cycles of energy minimization to eliminate close contacts between atoms. As in our prior studies (43–45), the program Discover (version 2.8, Biosym Technologies) was used for energy minimization with supplied constant value force field.

Alternative models were also generated by searching the crystallographic data base for loops of the same size as the CDR I region. The spatially conserved Cartesian positions at the NH_2- and COOH-terminal regions of CDR I were held fixed in the search procedure. A Cartesian distance matrix was constructed for combinations of the residues on the NH_2- and COOH-terminal regions of the CDR I and compared to a precalculated Cartesian distance matrix data base of high resolution protein structures (46). The 20 best matches were examined using the program Insight II and appropriate choices were made based upon similarities in chiralities of side chains at the junctures of the CDR I loop. The choice was spliced into the template using the program Insight II. Two alternative models were constructed using this approach. The one involved splicing a loop identified in the immunoglobulin Fc fragment 1Fc2 (*Model 2*). The other involved the heavy chain CDR I of 50.1 (*Model 3*). It is well known that light chains can adopt heavy chain conformations in the absence of heavy chain (47). The CDR I of the heavy chain 50.1 was spliced into the template.

TABLE II
Sequences of CDR-derived peptides

Designation	Sequence	Molecular mass
		Da
CDR I peptide	CRASKSVSSSGYSYMHWYQQ	2354
CDR II peptide	YLASNLESGVPPRFSGS	1779
CDR III peptide	CQHSRELPWTFGGGTRLEIKR	2470
Control peptide	SQGRNCSTNDSPL	1377

The alternative structures were mutated to the 23.2 sequence and the structures energy minimized. These models are presented in Fig. 6.

Prior studies investigating the epitope on GM-CSF recognized by 126.213 by mutagenesis (24) implicated residues 77–83, located on the C helix of GM-CSF. Peptide mapping studies of this antibody suggest recognition by the B and C helices as well as an epitope representing the first β strand, which are all structurally adjacent.[2] Analysis of the 23.2 models suggests a structural basis for mimicry of this site. This is shown in Fig. 6. All three models center our attention on residues Thr^{57}, Glu^{60}, and Lys63 of GM-CSF. The proximity of Lys^{63} and Glu^{60} suggest a charge-charge interaction. In all three models, these 2 residues are mimicked by Arg^{24} and Asp^{74} of 23.2. For Models

[2] VonFeldt, J. M., Monfardini, C., Fich, S., Rosenbaum, H., Kieber-Emmons, T., Williams, R. M., Kahn, S. A., Weiner, D. B., and Williams, W. V. (1995) *Pept. Res.*, in press.

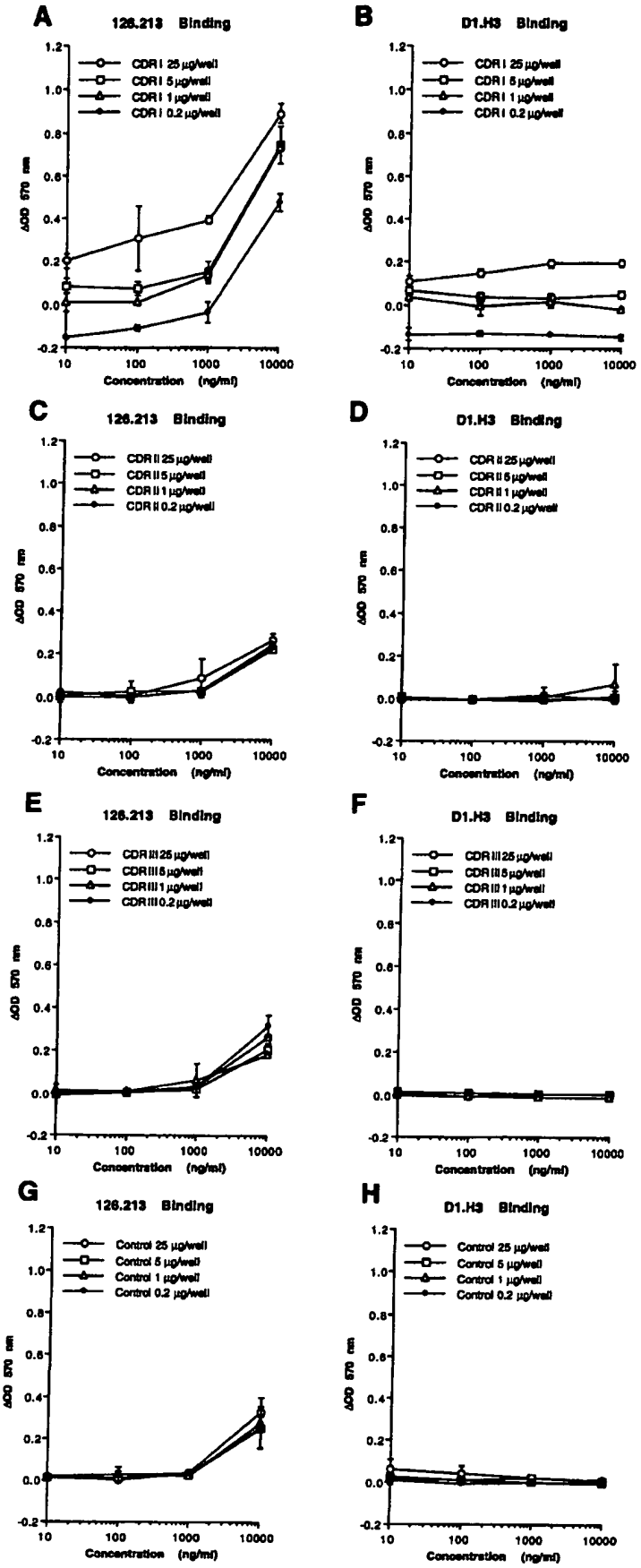

FIG. 7. **Binding of 126.213 to synthetic peptides derived from the 23.2 sequence.** Binding was performed by ELISA assay as described under "Materials and Methods." The values shown are A_{490} nm binding to the peptides at the concentration noted minus A_{490} nm binding to BSA-coated control plates. Results are compared for 126.213 *versus* an isotype matched control mAb (D1.H3) specific for a peptide derived from the hamster β-adrenergic receptor. The mean ± S.D. of triplicate wells is shown for increasing amounts of purified 126.213 added. *A* and *B*, binding to CDR I peptide. *C* and *D*, binding to the CDR II peptide. *E* and *F*, binding to the CDR III peptide. *G* and *H*, binding to the control peptide. The mAbs used were: 126.213 in *A*, *C*, *E*, and *G* and D1.H3 in *B*, *D*, *F*, and *H*.

1 and 3, the mimicry suggests similar orientations of 23.2, while for Model 2 the structure is rotated ~90°. The other GM-CSF residues mimicked include: Thr^{57} mimicked by Ser^{71} in Models 1 and 3, and by Thr^{76} in Model 2; Lys^{74} mimicked by Arg^{24} in Models 1 and 3, and Lys^{27} in Model 2; Thr^{78} and Ser^{82} mimicked by Ser^{28} in Models 1 and 3, and by Ser^{32} and Tyr^{34} in Model 2; Lys^{85} mimicked by Lys^{27} in Models 1 and 3, and by Ser^{56} in Model 2; and Glu^{93} mimicked by Ser^{30} and Ser^{32} in Model 1, and by Ser^{31} in Model 3. Thus, while sequence similarity between GM-CSF and 23.2 is quite low, structural similarity is suggested centered on the B and C helices of GM-CSF and the 23.2 CDR I.

Binding and Bioactivity of CDR Peptides—To further investigate the basis for mimicry by 23.2, synthetic peptides were developed based on the 23.2 CDR sequences. These are shown in Table II. The CDR peptides were used in an ELISA assay to determine binding by 126.213 (Fig. 7). Binding to the CDR II and CDR III peptides was not higher than binding to the control peptide used, although it was higher than the isotype matched control mAb used (Fig. 7). However, the CDR I peptide was bound at higher levels than the other CDR peptides and the control peptide, and was bound by 126.213 but not the control mAb. Additional studies using a competitive ELISA indicate that this peptide blocks GM-CSF binding by 126.213 (data not shown). This suggests that the CDR I region of 23.2 is the major recognition site for 126.213.

The ability of these peptides to compete with GM-CSF for binding to HL-60 cells was examined using a radioreceptor assay. HL-60 cells were preincubated with peptides prior to the addition of ^{125}I-GM-CSF and specific binding determined in the presence of excess unlabeled GM-CSF. A representative experiment is shown in Fig. 8. Increasing amounts of CDR I peptide were able to specifically inhibit GM-CSF binding in a dose-dependent manner, while CDR II and CDR III peptides did not demonstrate any specific binding inhibition. Thus, the CDR I peptide antagonizes ^{125}I-GM-CSF binding to HL-60 cells, suggesting interaction of this peptide with the GM-CSFR.

The bioactivity of these peptides was assessed by their effect on GM-CSF-dependent cellular proliferation. This was compared with their effect on interleukin-2-dependent proliferation by the CTLL cell line, to control for nonspecific toxic effects. The results are shown in Fig. 9. At the concentrations used, none of the peptides were toxic to CTLL cells with the exception of the CDR III peptide at 2 mg/ml. The CDR II peptide had no inhibitory effect on either cell line. In contrast, the CDR III and CDR I peptides inhibited GM-CSF-dependent cellular proliferation. For the CDR III peptide, the IC_{50} was ~1 mg/ml (approximately 400 μM), while for the CDR I peptide, it was ~50 μg/ml (approximately 21 μM). These data indicate that the CDR I peptide is a specific antagonist of GM-CSF-dependent cellular proliferation in a micromolar concentration range.

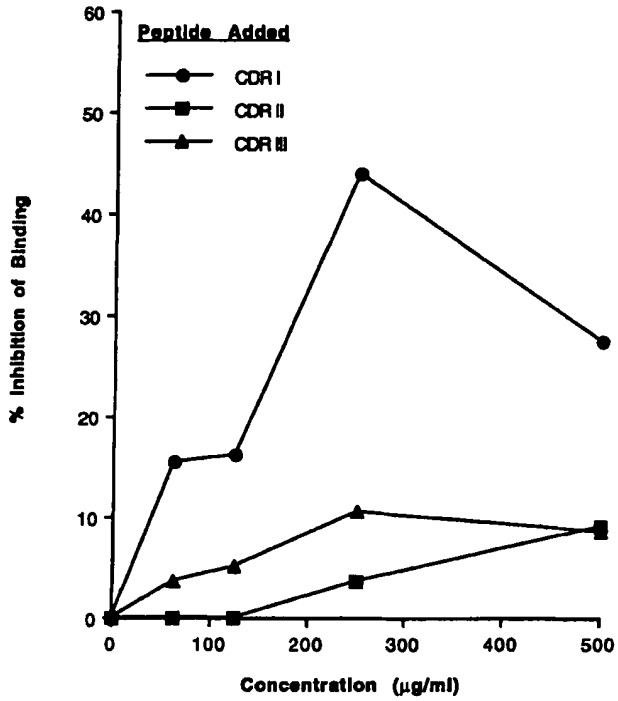

FIG. 8. **Inhibition of ^{125}I-GM-CSF binding to HL-60 cells by CDR peptides.** The radioreceptor assay was performed as noted under "Materials and Methods," using 10^6 HL-60 cells. The cells were preincubated with peptides at varying dilutions for 60 min at room temperature prior to the addition of ^{125}I-GM-CSF. The specific proportion of cpm bound was determined by subtracting the proportion of cpm bound under identical conditions in the presence of saturating amounts (50 nM) of unlabeled GM-CSF. The standard deviation of this assay was ~10% on multiple determinations. The percent inhibition of binding is shown *versus* increasing amounts of peptides.

DISCUSSION

GM-CSF-Receptor Interactions—GM-CSF activity is mediated by binding to specific cellular receptors (GM-CSFR) which belong to a recently described supergene family (38, 49–54). The high affinity GM-CSFR is comprised of an α chain (GM-CSFRα) specific for GM-CSF (38), and a β chain (β_c), which can also associate with the IL-3 and IL-5 receptor α chains (52). The GM-CSFRα imparts specificity to the interaction with GM-CSF, and when expressed without β_c is able to bind GM-CSF, albeit with lower affinity than the heterodimeric receptor (55). The high affinity receptor (GM-CSFRα and β_c) appears to be the signal transducing unit (56, 57), with a sequential binding of GM-CSF to GM-CSFRα followed by binding to β_c postulated. The formation of a ternary complex of GM-CSF with GM-CSFRα and β_c implies that more than one site on GM-CSF is needed for receptor binding and bioactivity.

GM-CSF binding and bioactivity have been analyzed at a molecular level. Mutagenesis studies implicate the first (A) helix in binding of GM-CSF to the high affinity GM-CSFRα/β_c complex, but not to the low affinity receptor (GM-CSFRα alone) (55, 58, 59). This is illustrated most strikingly by studies using mutants of residue Glu^{21} of GM-CSF, which inhibit binding of GM-CSF to the low affinity receptor, but display little activity in inhibiting binding to the high affinity receptor (58). Based on these experiments, it has been proposed that the first α helix of GM-CSF is responsible for binding to β_c (59). Murine and human GM-CSF display species specificity and are not cross-reactive. As the substitutions are scattered throughout the molecule, it was possible to swap regions of murine and human GM-CSF to locate sites critical for receptor interaction (37). These studies indicated a critical role for amino acids 21–31 and 77–94 in mediating the activity of human GM-CSF, suggesting that the second site may be involved in binding to the GM-CSFRα. However, other potential GM-CSFRα interaction sites have also been suggested in mutagenesis studies (60–62), mapping of neutralizing mAbs (24, 63–65), and synthetic peptide studies (48, 63, 66). Thus, in spite of considerable study, the GM-CSFRα interaction site(s) on GM-CSF remain incompletely characterized.

Recent studies from our group have used synthetic peptides, anti-peptide antisera, and neutralizing mAbs to map epitopes on GM-CSF critical for bioactivity.[2] The major findings were: a peptide derived from the sequence of the A helix (residues 17–31) and antibodies to this peptide inhibited GM-CSF-dependent cellular proliferation; a peptide comprising portions of the B and C helices (residues 54–78) was recognized by two

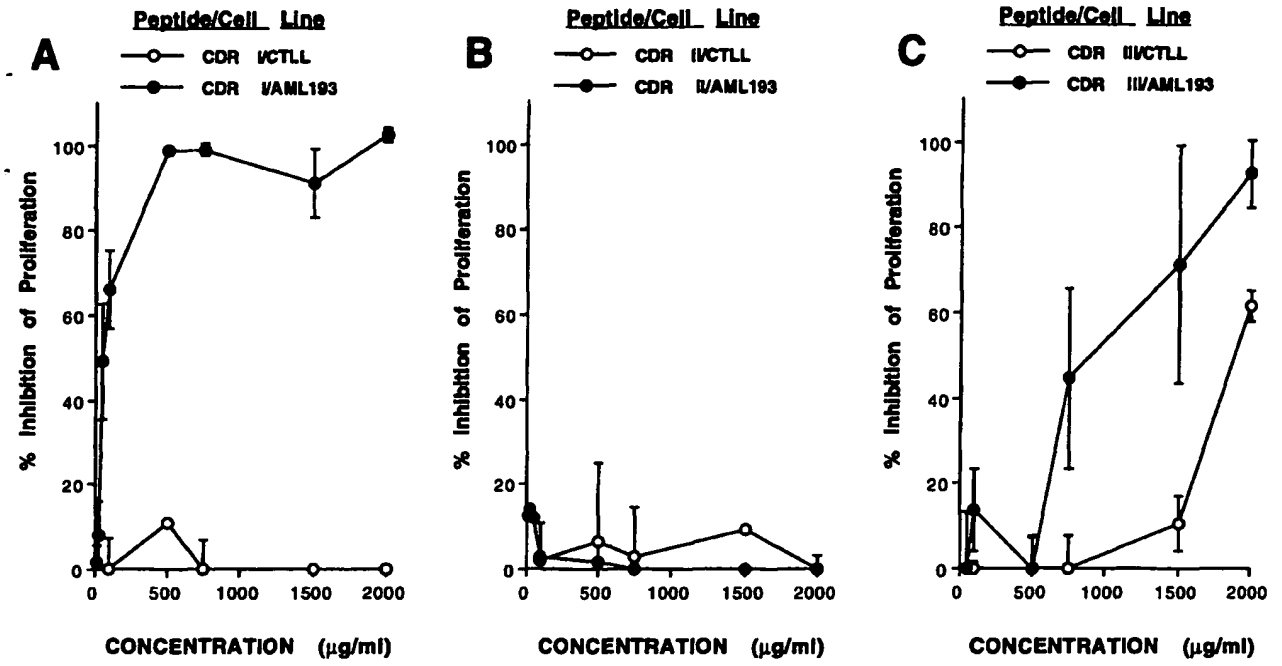

FIG. 9. **Inhibition of GM-CSF-dependent cell proliferation by peptides.** The proliferation assay was performed as noted under "Materials and Methods" on AML193 cells (GM-CSF-dependent) and CTLL cells (IL-2-dependent), in the presence or absence of increasing amounts of peptides as noted. Results from two experiments are combined, with the mean ± standard error percent inhibition of proliferation shown *versus* increasing peptide concentration. *A*, CDR I peptide; *B*, CDR II peptide; *C*, CDR III peptide.

neutralizing monoclonal antibodies (including 126.213) and exhibited biological antagonist activity. Other peptides were also bound by 126.213 corresponding to residues 78–99 and 31–54, but were not specific antagonists of GM-CSF bioactivity. These three peptides together constitute a "face" on GM-CSF centered on the B and C helices and opposite the A helix. Together with the prior studies noted above, these studies suggest two binding sites on GM-CSF important in receptor binding: the A helix which likely interacts with β_c, and the opposite face centered on the B and C helices which we propose interacts with the GM-CSFRα. The ability of synthetic peptides corresponding to these epitopes to specifically inhibit GM-CSF bioactivity strongly supports their role in receptor interaction.

GM-CSF Mimicry by Recombinant Antibody Light Chain—The studies described here suggest that the 23.2 rAb fragment mimics a binding site on GM-CSF involved in interaction with the GM-CSFRα. 23.2 was selected to bind to the antigen binding idiotopes of the neutralizing mAb 126.213, which competes with a soluble form of the GM-CSFRα for binding to GM-CSF. 23.2 displays several features characteristic of an "internal image" of the antigen including competition with GM-CSF for binding to 126.213 and to HL-60 cells (Figs. 3 and 4). The sequence similarity of 23.2 with GM-CSF noted, while weak, is spread out over residues 53–98, which comprises the B and C helices as well as the BC interhelical loop, and represent one "face" of the GM-CSF molecule (42). The weak sequence similarity seen led to the development of molecular models of 23.2 to investigate a potential structural basis for the mimicry observed (Fig. 6). This suggests mimicry of specific residues on the GM-CSF B and C helices by specific residues in the 23.2 CDR I, CDR II, and FR3 regions. Synthetic peptides corresponding to the 23.2 CDR regions were developed and evaluated. This analysis led to the observation that the CDR I peptide is recognized by 126.213 (Fig. 7) and is a biological and receptor antagonist of GM-CSF (Figs. 8 and 9). The CDR I region of 23.2 contributes most of the residues implicated in the structural analysis. The activity of the CDR I peptide confirms the importance of these residues and suggests that this peptide interacts with the GM-CSFRα, functioning as a receptor antagonist.

In prior studies, we described the molecular basis for antibody mimicry of a viral hemagglutinin (9–12). Other groups have applied this technology to platelet fibrinogen receptor (14), the thyroid-stimulating hormone receptor (15), and epitopes on the hepatitis B surface antigen (17). Monoclonal antibodies were utilized in these studies as mimics and to derive sequence information. The studies presented here are the first to suggest that recombinant antibodies can be similarly employed to develop alternative ligands. The prior studies of antibody mimicry in general described mimicry of structures either known or predicted to represent reverse turns. As antibody CDRs are generally reverse turns, the ability of antibody CDRs to mimic other reverse turn regions does not necessarily imply that CDRs can mimic amino acid residues presented by other diverse backbone geometries. The epitopes involved in this study are largely α helical in nature. In spite of this, molecular modeling of this epitope suggests a structural basis for mimicry as noted above. This indicates that antibody mimicry of amino acid arrays on helical regions can be understood on a molecular-structural level. The application of recombinant antibody technology to development of such mimics should broaden the applicability of alternative ligand development in the analysis of active site structures.

Acknowledgments—We wish to thank L. Marie for her helpful comments and S. Fish for her most kind assistance.

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Design of Bioactive Peptides Based on Antibody Hypervariable Region Structures

DEVELOPMENT OF CONFORMATIONALLY CONSTRAINED AND DIMERIC PEPTIDES WITH ENHANCED AFFINITY*

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William V. Williams‡‖, Thomas Kieber-Emmons‡§, Joan VonFeldt‡, Mark I. Greene¶, and David B. Weiner‡§

From the Departments of ‡Medicine and ¶Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, the §Wistar Institute of Anatomy and Biology, and the ‖Childrens Hospital of Pennsylvania, Philadelphia, Pennsylvania 19104-4283

The variable regions of antibody molecules bind antigens with high affinity and specificity. This binding is imparted largely by the hypervariable portions of the variable region. Hypervariable regions typically fold into reverse turn or loop structures. Peptides derived from antibody hypervariable region sequences can bind antigens with similar specificity, albeit with markedly lower affinity. In this study, cyclic and dimeric peptide analogs of an anti-idiotypic/antireceptor antibody hypervariable region were developed. This antibody (87.92.6) binds to reovirus type 3 receptors on cells as well as to a neutralizing anti-reovirus type 3 monoclonal antibody (9B.G5). The cyclic peptides were utilized to probe the optimal conformation for binding to both the receptor and 9B.G5. By dimerizing or constraining the conformation of these peptides, higher affinity binding was produced. By utilizing several different cyclic peptides, the optimal conformation for binding was established. The conformationally optimized cyclic peptide possessed >40-fold higher affinity for the receptor and the idiotype than the linear analog. This study suggests that conformationally constrained and dimeric peptides derived from antibody hypervariable loop sequences can bind antigens (including receptors) with reasonable affinity.

Antibodies utilize complementarity-determining regions (CDRs)[1] of their variable domains to bind antigens with high affinity and specificity (1-5). Comparison of the CDRs from distinct antibodies reveals that they are hypervariable in amino acid sequence. The specific amino acids of the CDRs determine the binding specificity of an antibody molecule. Structurally, CDRs predominantly fold into reverse turns and occasionally helix-like structures (1, 6, 7). CDRs often contain contact residues with the antigen, although some contact residues are located within framework regions as well (8). The binding strategy utilized by these antibodies can be analyzed to develop synthetic peptides with similar binding properties. This has been accomplished in a number of systems where synthetic peptides have been derived directly from the amino acid sequences of CDRs and demonstrated to have binding properties similar to those of the intact antibody (9, 10). Examples include CDR-derived peptides that inhibit idiotype/anti-idiotype interactions, interact with specific antigens, bind to cellular receptors, and possess biological activity (10-14).[2] This strategy provides analysis of intermolecular interactions involved in binding and can lead to development of novel binding moieties with predictable activities.

We have been studying an antireceptor antibody that is also anti-idiotypic to a neutralizing anti-reovirus type 3 monoclonal antibody. The antibody 87.92.6 binds to both the reovirus type 3 receptors (Reo3R) on cells and to the neutralizing anti-reovirus type 3 monoclonal antibody 9B.G5 (16, 17) (see Fig. 1 for interactions). The CDRs II of both the light and heavy chain variable regions of 87.92.6 were demonstrated to possess amino acid sequence similarity to a determinant on reovirus type 3 hemagglutinin (HA3 = cell attachment protein of the virus) (18). Synthetic peptides were developed based on 87.92.6 light chain variable CDR II (V_L peptide) and heavy chain variable CDR II (V_H peptide). These peptides corresponded exactly to amino acids 39-54 of the 87.92.6 light chain variable region for the V_L peptide and amino acids of the heavy chain variable region for the V_H peptide. In a series of studies, the V_L peptide was demonstrated to interact with both 9B.G5 and the Reo3R in a specific manner. Binding of some forms of the V_L peptide to either 9B/G5 or the Reo3R inhibited the binding of both 87.92.6 and reovirus type 3 (19).[2] In contrast, the V_H peptide bound to 9B.G5 in some assays, but not to the Reo3R.

In other studies, we have demonstrated that the V_L peptide, but not the V_H peptide, has biological activity similar to that of 87.92.6, that is, the V_L peptide inhibits concanavalin A-induced murine lymphocyte mitogenesis (14). In addition, the V_L peptide can be rendered dimeric by addition of an amino-terminal cysteine residue. This dimeric form of the V_L peptide reproduces several activities of 87.92.6, including inhibition of murine fibroblast growth and down-modulation of the Reo3R (14), alterations in Schwann cell growth and function (19), and changes in the growth and differentiation of oligo-

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[1] The abbreviations used are: CDRs, complementarity-determining regions; Reo3R, reovirus type 3 receptor; PBS, phosphate-buffered saline; RIA, radioimmunoassay; BSA, bovine serum albumin.

[2] W. Williams, T. Kieber-Emmons, D. Rubin, M. Greene, and D. Weiner, *J. Biol. Chem.*, submitted for publication.

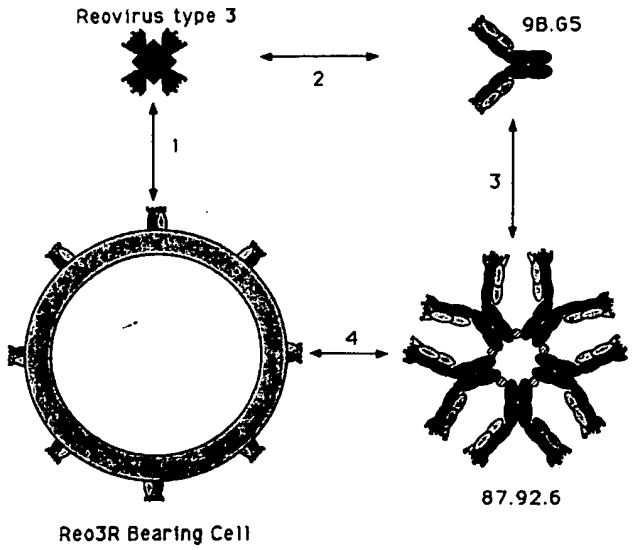

FIG. 1. **Interactions among reovirus type 3, its receptor, and antibodies.** Reovirus type 3 binds specific receptors (designated Reo3R) on receptor-bearing cells (*interaction 1*). The site on reovirus type 3 hemagglutinin (HA3) that binds the receptor is bound by 9B.G5 (murine IgG2a), which neutralizes viral infectivity (2). 9B.G5 in turn is bound by 87.92.6 (murine IgM) (3). 87.92.6 binds to the Reo3R similarly to reovirus type 3 (4).

dendrocytes (20). These studies indicated that it is possible to develop receptor-binding peptides based on the structure of antibody CDRs.

In the initial studies of the V_L peptide, the affinity of interaction between the unmodified V_L peptide and both 9B.G5 and the Reo3R was low. This low affinity of interaction may have been due in part to either enthalpic or entropic considerations. Short peptides are thought to have a disordered solution conformation without a dominant folding pattern. Thus, for binding to occur, the peptides must assume an appropriate conformation. If the low energy solution structure is not the binding mode structure, then there is an enthalpic component for the conformational adjustment. In addition, the peptide must overcome inherent entropy in such a process. These aspects lower the net free energy of binding, making a high affinity interaction difficult to obtain. To decrease these energy considerations, we have developed conformationally constrained analogs of the V_L peptide. These peptides help to orient the contact residues in specific relative conformations. By utilizing several different cyclic peptides, the optimal conformation of binding can be evaluated. An additional strategy is to increase the avidity of binding, by creating dimeric peptides. Such peptides should also display a higher apparent affinity than the monomeric forms. The properties of these peptides are described herein.

MATERIALS AND METHODS

Peptides—All peptides were synthesized by solid-phase methods, deprotected, and released from the resin utilizing anhydrous HF. Peptides were lyophilized and further purified by high performance liquid chromatography utilizing a TSK 3000 column and again lyophilized. Purity was assessed by high performance liquid chromatography utilizing a Waters C_8 column and a 0–70% acetonitrile gradient. All peptides were >90% pure. Peptides (containing internal cysteine residues) were oxidized for experiments by dissolving them at ≤2 mg/ml in distilled water and stirring them overnight exposed to the air at 23 °C. The peptides had no free sulfhydryls following this procedure by Ellman determination.

Virus and Cell Lines—Purified reovirus type 3, prepared as previously described (10), was kindly provided by D. H. Rubin (Philadelphia Veterans Administration Hospital). Purified virus was radioiodinated by the chloramine-T method (10). Murine L cells (fibroblasts) were grown in Joklik's modified minimal essential medium with 10% fetal calf serum and added sodium bicarbonate, Pen/Strep, and L-glutamine (all from GIBCO), whereas murine R1.1 cells (thymoma) were grown in RPMI 1640 medium (GIBCO) with 10% fetal calf serum and added Pen/Strep and L-glutamine.

Monoclonal Antibodies—Neutralizing anti-reovirus type 3 monoclonal antibody 9B.G5 (murine IgG2a,κ) or isotype-matched monoclonal antibody 11 were isolated from culture supernatant by 50% ammonium sulfate precipitation; dialyzed against phosphate-buffered saline (PBS); bound to a staphylococcal protein A column (Sigma), eluted with 0.1 M citric acid, pH 3.5; neutralized with 0.5 M Tris-HCl, pH 8.5; dialyzed against PBS; and concentrated on an Amicon protein concentrator. Monoclonal antibodies 87.92.6 and HO13.4 (both murine IgM,κ) were grown as ascites or culture supernatants from hybridoma cells. For 87.92.6, ascites was filtered and stored at −70 °C prior to use. This was necessary due to the observed instability of this antibody when stored in purified form. For HO13.4, the antibody was immunoaffinity-purified over a goat anti-mouse IgM column, eluted with 3.5 M $MgCl_2$, and dialyzed against PBS as previously described (10). The concentration of HO13.4 was adjusted to give similar binding on flow cytometry analysis as an identical volume of 87.92.6 ascites.

Determination of Free Sulfhydryls in Peptides (Ellman Determination)—Peptides dissolved in H_2O at 2 mg/ml were added at 5, 10, or 20 μl to 10 mM $NaPO_4$, pH 7.0, for a final volume of 1 ml. To this was added 6 μl of 2,2'-bisazidothiobenzoic acid (Sigma) in 50 mM $NaPO_4$, pH 8.0. This was allowed to react for >3 min, and the absorbance at 420 nm was then determined. Percent loss of sulfhydryls was determined by the formula: $100 \times ((A_{420}$ untreated peptide $- A_{420}$ oxidized peptide$)/A_{420}$ untreated peptide).

Evaluation of Peptide Cyclization—Peptide cyclization *versus* oligomerization was evaluated by gel exclusion chromatography. A 100 × 1-cm column (Bio-Rad) was constructed with Sephadex G-25 (superfine; Pharmacia LKB Biotechnologies, Inc) and equilibrated with distilled/deionized water. This was utilized along with an inflow peristaltic pump (2232 Microperpex S), ultraviolet monitor (2138 Uvicord S), fraction collector (2212 Helirac), and chart recorder (2210 1-channel recorded, all from Pharmacia LKB Biotechnology Inc.). Peptides were diluted in distilled/deionized H_2O to 1 mg/ml, and 500 μl was loaded directly on the column bed and allowed to run into the column. This was followed by several small volumes of distilled/deionized H_2O to wash in the sample, and the column was then run at a constant rate of 0.5 ml/min. Fractions of 40 drops were collected. On this system, bovine serum albumin eluted in fractions 8 and 9 and L-tyrosine in fractions 17–20. The oxidized V_LSH peptide eluted in fractions 11 and 12, whereas the unmodified V_L peptide eluted in fractions 13 and 14. To assess cyclization *versus* oligomerization, peptides were run following reduction with β-mercaptoethanol (100 × molar excess of β-mercaptoethanol added to peptides diluted to 1 mg/ml in distilled/deionized H_2O and sealed under N_2 for 1 h) and were then run on the Sephadex G-25 column following equilibration of the column with 5×10^{-6} M β-mercaptoethanol and run in the same buffer. The fractions of elution of reduced peptides was compared with the fractions of elution of peptides oxidized overnight to assess cyclization *versus* oligomerization.

Radioimmunoassay (RIA)—RIA plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with peptides by evaporation of varying amounts of peptides in distilled water overnight at 37 °C. The wells were washed with PBS, blocked with 2% bovine serum albumin (BSA) in PBS with 0.1% NaN_3, and washed with PBS. Partially purified 9B.G5 ($(NH_4)_2SO_4$ precipitate dialyzed against PBS) was added at varying dilutions for >1 h at 37 °C. The wells were washed with PBS, and 50,000–100,000 cpm of ^{125}I-labeled goat anti-mouse light chain (anti-κ and anti-λ (Sigma) iodinated by chloramine T) was added per well in 1% BSA in PBS for 1–2 h at 37 °C. The wells were decanted and washed extensively, and the counts/minute bound were determined. The specific counts/minute bound were determined by subtracting the counts/minute bound to uncoated wells from the counts/minute bound to peptide-coated wells. The low counts/minute bound obtained represented specific and reproducible binding based on additional assays (data not shown).

Competitive RIA—RIA plates were coated with staphylococcal protein A (Sigma) by incubation of a 50 μl/well concentration of a 5 μg/ml solution overnight at 4 °C. The wells were washed with PBS and blocked with 2% BSA, PBS, 0.1% NaN_3; and purified 9B.G5 or isotype-matched control monoclonal antibody A11 was adsorbed to the wells by incubation of 50 μl of a 10 μg/ml solution (purified

antibody) in 1% BSA, PBS, NaN_3 for 1–2 h at 37 °C. The wells were washed; and competitors were added at various concentrations in 100 μl of 0.5% BSA, 0.45% NaCl, 0.05% phosphate buffer, pH 7.2, for 1 h at 37 °C. ^{125}I-Labeled reovirus type 3 (5–10 × 10^5 cpm/well) or unlabeled antibody (87.92.6 or isotype-matched monoclonal antibody E4.49.2) at a 1:100 dilution of ascites in 1% BSA, PBS, 0.1% NaN_3 was added for an additional 30–45 min at 23 °C. For wells incubated with 87.92.6, the wells were washed with PBS, and ^{125}I-labeled goat anti-mouse Ig was added for an additional 60 min at 37 °C. The wells were washed extensively, and the counts/minute bound were determined. For reovirus binding, the specific counts/minute bound were determined by subtracting the counts/minute bound to monoclonal antibody 11-coated wells from the counts/minute bound to 9B.G5-coated wells. For 87.92.6 binding, specific binding was determined by subtracting the counts/minute bound following incubation with E4.49.2 ascites from the counts/minute bound following incubation with equivalent amounts of 87.92.6 ascites. Percent inhibition of binding was determined by the formula: ((specific cpm bound without inhibitor − specific cpm bound with inhibitor)/specific cpm bound without inhibitor) × 100.

Inhibition of Viral Binding to Cells—The cells were centrifuged and washed twice with 1% BSA, PBS, 0.1% NaN_3; and 5 × 10^4 L cells or 1.25 × 10^6 R1.1 cells in 50 μl were distributed in 2% BSA, PBS, NaN_3-blocked RIA wells. For peptide studies, 50 μl of inhibitor was added in distilled H_2O to the cells. Following a 30-min incubation, L cells and ^{125}I-labeled reovirus type 3 were combined for an additional 30 min at 37 °C. The cells were centrifuged down and washed three times in ice-cold PBS, and the specific counts/minute bound were determined as noted above. Percent inhibition of binding was calculated by the formulas noted above.

Flow Cytometry Analysis—The ability of peptides to inhibit antibody binding to cells was determined by preincubation of the cells with varying amounts of inhibitor (in 100 μl of distilled H_2O) for 30 min to 1 h at 23 °C. Cells (either L or R1.1 cells) were washed with 1% BSA, PBS, 0.1% NaN_3 and resuspended at 10^7/ml. Cells (100 μl) were then added in 1% BSA, PBS, 0.1% NaN_3; and incubation was continued for 20–30 min. Antibodies (5 or 10 μl) were added for an additional 20 min at 23 °C. Ice-cold 1% BSA, PBS, 0.1% NaN_3 was added; and the cells were centrifuged and washed prior to counterstaining with a 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse Ig (Fisher) in 1% BSA, PBS, 0.1% NaN_3. The cells were washed twice, and fluorescence intensity was determined as previously described (10, 14). Inhibition of binding was calculated as noted above with Δ mean channel number utilized in place of counts/minute.

RESULTS

Peptide Cyclization—One way to evaluate the optimal folding conformation of the V_L peptide is reflected by the ability of cysteine-containing variants to cyclize. If the cysteine residues are placed in various positions on one or the other side of a predicted reverse turn, the residues placed in the most energetically favorable locations for assuming a reverse turn structure should also form disulfide bridges (become oxidized) most rapidly. This was established utilizing several cysteine-containing peptides as outlined in Table I. These peptides were subjected to oxidation by agitating a solution (2 mg/ml in 0.1 M $NaHCO_3$) at 37 °C for varying periods of time exposed to air. The disappearance of free sulfhydryls was quantitated by Ellman determination as described above, and percent loss of sulfhydryls with time was calculated. The results are shown in Fig. 2.

As noted, $V_LC_8C_{16}$ and $V_LC_9C_{16}$ had the most rapid loss of sulfhydryls in this assay, whereas $V_LC_{10}C_{16}$ demonstrated a reduced rate of loss of free sulfhydryls. This implies that the $V_LC_{10}C_{16}$ peptide forms intramolecular disulfide bridges more slowly than the other two peptides. One explanation of this finding is that the corresponding cyclic conformation of $V_LC_{10}C_{16}$ may be energetically more costly to assume than that of $V_LC_8C_{16}$ or $V_LC_9C_{16}$.

The oxidation of these peptides did not necessarily imply that cyclic peptide formation was taking place as intermolecular disulfide bridges also may have been forming. This issue was addressed by examining reduced (β-mercaptoethanol-treated) *versus* oxidized variants of these peptides by size exclusion chromatography utilizing a Sephadex G-25 (superfine) column (Fig. 3).

The $V_LC_8C_{16}$ and $V_LC_9C_{16}$ peptides largely eluted in similar fractions in both the oxidized and reduced states, implying that they remained chiefly as monomers following oxidation. In fact, the major peaks of all the oxidized peptides took slightly longer to elute than the corresponding reduced peptide, suggesting a more compact form. An additional peak appeared for $V_LC_{10}C_{16}$ following oxidation that migrated more rapidly than the predominant peak seen in the reduced form. This indicates that $V_LC_{10}C_{16}$ is forming higher molecular weight species following oxidation, likely due to the formation of intermolecular disulfide bridges. In contrast, $V_LC_8C_{16}$ and $V_LC_9C_{16}$ did not form significant amounts of higher molecular weight species, implying less intermolecular disulfide bridge formation for these peptides. These data suggest that $V_LC_8C_{16}$ and $V_LC_9C_{16}$ more readily fold into an appropriate conformation for intramolecular disulfide bond formation compared with $V_LC_{10}C_{16}$ and that the slower rate of oxidation of $V_LC_{10}C_{16}$ was indicative of intermolecular bond formation in addition to intramolecular bond formation.

In addition to these analyses, the oxidized peptides were subjected to amino acid analysis at the Wistar Institute Peptide Analysis Facility by David Speicher following hydrolysis and analysis by high performance liquid chromatography (C_{18} column). These studies indicated an average of <0.1 cysteic acid residue/peptide, implying that the major oxidized form was cystine. Coupled with the observation that the majority of the oxidized peptide species migrated at similar or slower rates on gel exclusion compared with the reduced forms, this implies that oxidation resulted chiefly in intramolecular disulfide bonds *versus* intermolecular disulfide bond formation. Together, these results indicate that the major species of the peptides used in these experiments represent cyclic peptides and not some other form.

Structural Analysis of Cyclic Peptides—The predicted structures of these presumed cyclic peptides is shown in Fig. 4. These model structures were derived by energy minimization of the constrained peptides utilizing the energy parameters

TABLE I

Cyclic peptide variants of the V_L peptide

The sequences of the indicated peptides are shown in three-letter code. Other peptides utilized included the V_H peptide (Cys-Gln-Gly-Leu-Glu-Trp-Ile-Gly-Arg-Ile-Asp-Pro-Ala-Asn-Gly) and a control peptide (Thr-Tyr-Arg-Tyr-Pro-Leu-Glu-Leu-Asp-Thr-Ala-Asn-Asn-Arg) matched for approximate length and hydrophobicity.

Designation	Sequence
V_L	Lys-Pro-Gly-Lys-Thr-Asn-Lys-Leu-Leu-Ile-Tyr-Ser-Gly-Ser-Thr-Leu-Gln
V_LSH	Cys-Lys-Pro-Gly-Lys-Thr-Asn-Lys-Leu-Leu-Ile-Tyr-Ser-Gly-Ser-Thr-Leu-Gln
$V_LC_8C_{16}$	Lys-Pro-Gly-Lys-Thr-Asn-Lys-Cys-Leu-Ile-Tyr-Ser-Gly-Ser-Thr-Cys-Gln
$V_LC_9C_{16}$	Lys-Pro-Gly-Lys-Thr-Asn-Lys-Leu-Cys-Ile-Tyr-Ser-Gly-Ser-Thr-Cys-Gln
$V_LC_{10}C_{16}$	Lys-Pro-Gly-Lys-Thr-Asn-Lys-Leu-Leu-Cys-Tyr-Ser-Gly-Ser-Thr-Cys-Gln

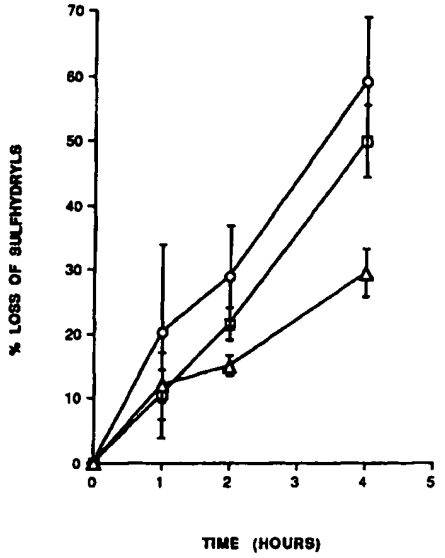

FIG. 2. **Rate of loss of sulfhydryls for various peptides.** Peptides (see Table I) were agitated at 37 °C for varying periods of time, and loss of sulfhydryls was quantitated as described under "Materials and Methods." Percent loss of total evaluable sulfhydryls is shown as a function of time of oxidation. The mean ± S.D. of two separate assays is shown. □, $V_LC_9C_{16}$; ○, $V_LC_9C_{16}$; △, $V_LV_{10}V_{16}$.

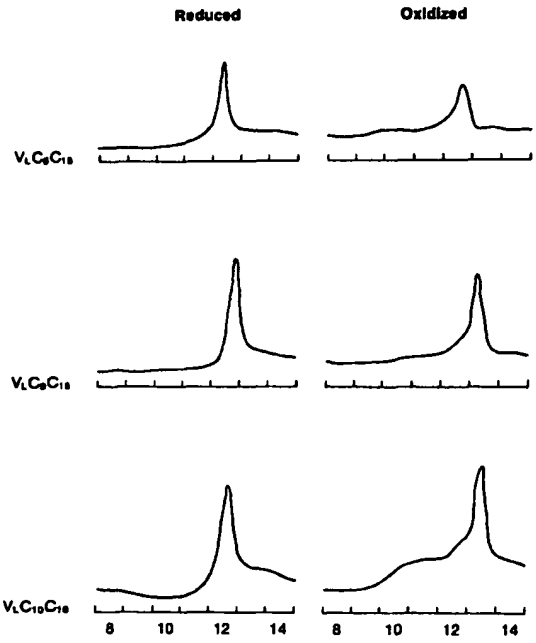

FIG. 3. **Size evaluation of oxidized peptides.** Peptides were reduced with a 10–100 × molar excess of β-mercaptoethanol (reduced) or were oxidized by agitation open to the air overnight (oxidized). These preparations were then run on a Sephadex G-10 (superfine) column, and the absorbance at 280 nm was measured. The A_{280} is shown *versus* fraction number for the preparations.

and functions of Kollman and co-workers (21). Models were initially constructed utilizing the geometry of the CDR2L of REI as previously described (10, 14). Residues corresponding to the cyclic analogs (Table I) were generated using the program INSIGHT® (Biosym Technologies), and the conformational properties of the analogs were examined using the program DISCOVER® (Biosym Technologies). The structures were minimized using a cutoff distance of 7.5 Å and a sliding scale dielectric constant. The root mean square difference between the V_L starting geometry and the cyclic analogs was calculated as a measure of the conformational differences in the backbone atoms of the structures. The root mean squares were $V_L/V_LC_{10}C_{16}$ (0.96) > $V_LC_9C_{16}$ (1.52) > $V_LC_8C_{16}$ (1.80). The root mean square differences would imply that the inclusion of the cysteine residues in the $V_LC_{10}C_{16}$ analog produced the closest structure to the previously modeled V_L structure (10). Comparison of these modeled cyclic peptides to the presumed binding mode for the V_L peptide[2] is currently underway.

Binding of 9B.G5 to Peptides—To assess the optimal conformation for binding of the cyclic V_L peptide analogs, they were utilized to coat RIA plates, and 9B.G5 was bound by standard RIA procedures. The results are shown in Fig. 5. As can be seen, the total counts/minute bound are low, but are reproducible and comparable to results from other investigators (22, 23) utilizing similar protocols. Binding to the $V_LC_9C_{16}$ peptide was higher than binding to the other cyclic V_L peptide analogs. This indicates that the $V_LC_9C_{16}$ peptide has higher binding to 9B.G5 on solid-phase RIA in comparison with the other cyclic peptides.

Inhibition of 9B.G5/87.92.6 Interaction by Peptides—Whereas the solid-phase RIA indicates a higher affinity of 9B.G5 to $V_LC_9C_{16}$ compared to the other cyclic peptides, it does not address the affinity of this interaction in solution. To investigate the optimal solution conformation for 9B.G5 binding, peptides were utilized to inhibit the 9B.G5/87.92.6 interaction in a liquid-phase assay (Fig. 1, *interaction 1*). As shown in Fig. 6, the results of this assay indicate that the $V_LC_9C_{16}$ peptide again demonstrates greater inhibition of this interaction compared with the other cyclic peptide variants. When compared with a linear analog of the V_L peptide (Fig. 7), the $V_LC_9C_{16}$ peptide displays an increased inhibition of binding. This suggests that the increased conformational stability of this cyclic peptide augments its binding affinity for 9B.G5. It is also demonstrated that a peptide derived from 87.92.6 heavy chain variable CDR II (V_H peptide) is able to inhibit the 9B.G5/87.92.6 interaction. Whereas this peptide inhibits the idiotype/anti-idiotype interaction, it does not significantly interact with the Reo3R (see below).

Inhibition of 9B.G5/Reovirus Type 3 Interaction by Peptides—To evaluate further the optimal conformation for cyclic peptide binding to 9B.G5 in solution phase, peptides were utilized to inhibit binding of ^{125}I-labeled reovirus type 3 to 9B.G5 in a similar assay (Fig. 1, *interaction 2*). The results are shown in Fig. 8. As can be seen, the $V_LC_9C_{16}$ peptide exhibited higher inhibition of binding than the other cyclic peptides in this assay. When compared with the linear V_L peptide and the dimeric V_LSH peptide in a separate set of assays (Fig. 9), the $V_LC_9C_{16}$ peptide demonstrates greater inhibition than the linear V_L peptide and similar inhibition on a molar basis as the dimeric V_LSH peptide.

Together, the data in Fig. 5–9 indicate that 9B.G5 has higher binding to the $V_LC_9C_{16}$ peptide compared with the other cyclic peptides and that this peptide is also the most potent inhibitor of 87.92.6 or reovirus type 3 binding to 9B.G5. This suggests that this cyclic peptide represents a preferred binding conformation to 9B.G5. This study also suggests that this conformation produces a higher affinity of binding compared with the linear analog.

Inhibition of Reo3R/87.92.6 Interaction by Peptides—To assess the interactions of the cyclic peptides with the Reo3R, they were utilized in a series of assays to inhibit binding of 87.92.6 or control antibodies to specific receptors (Fig. 1, *interaction 4*). As shown in Fig. 10, the $V_LC_9C_{16}$ peptide

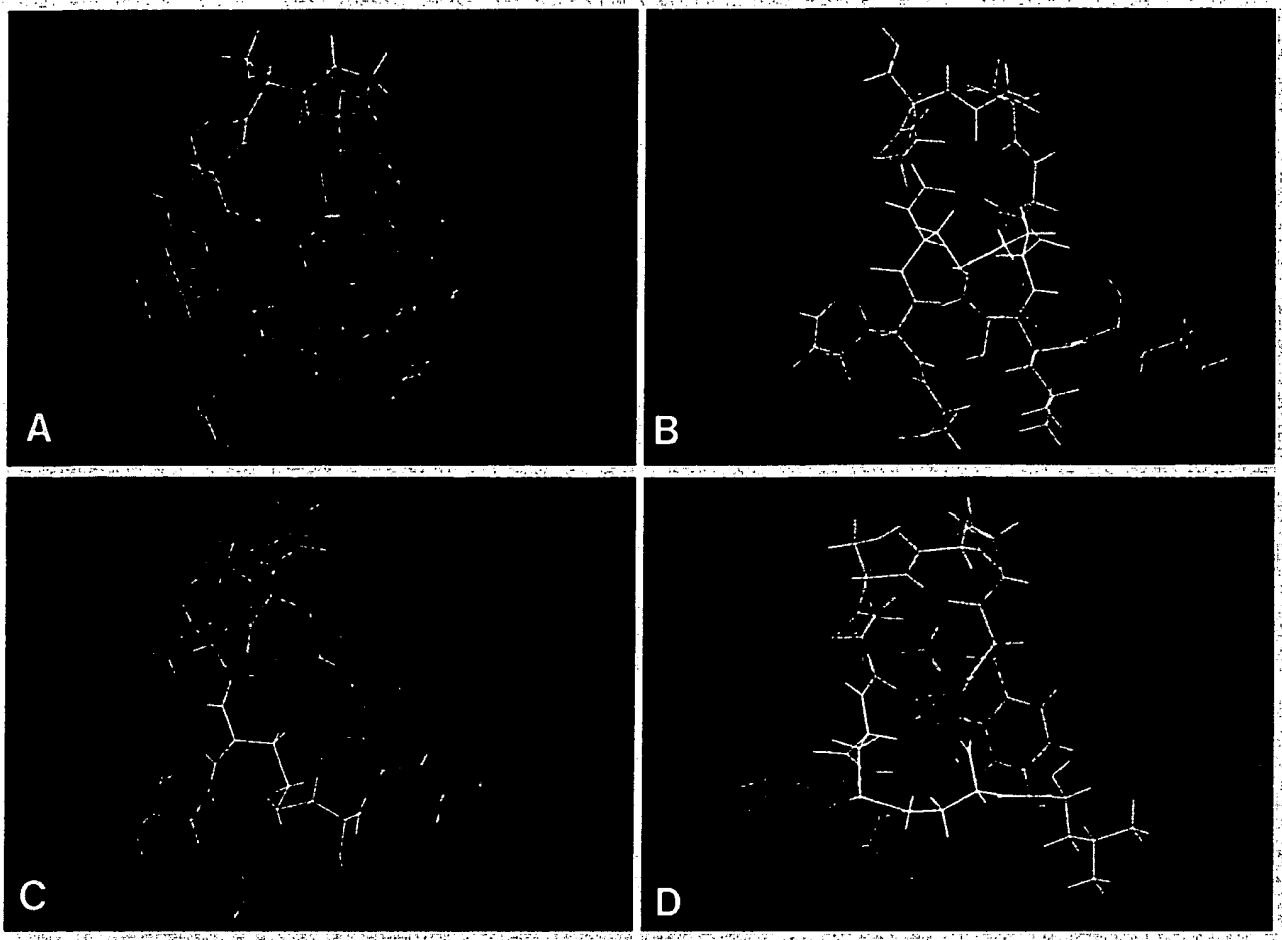

FIG. 4. **Molecular models of peptide reverse turn regions.** Model structures were derived as discussed in the text. *A*, model of the V_L peptide previously developed (10, 14); *B*, $V_LC_{10}C_{16}$ peptide; *C*, $V_LC_9C_{16}$ peptide; *D*, $V_LC_8C_{16}$ peptide. Color code: cysteine residues are in *yellow*, Leu^6 and Leu^{16} are in *red*, Leu^9 is in *green*, and Ile^{10} is in *purple*. All other residues are in *light blue*.

inhibits binding of 87.92.6 to murine L cells and R1.1 thymoma cells. In contrast, the linear V_H peptide has no effect on 87.92.6 binding, whereas the V_L peptide is a less effective competitor on L cells and ineffectual on R1.1 cells. Inhibition by the $V_LC_9C_{16}$ peptide is specific as binding of isotype-matched monoclonal antibody HO13.4 to Thy1.2 molecules is not inhibited by the $V_LC_9C_{16}$ peptide (Fig. 11). We have previously reported (10) that the V_L peptide does not inhibit binding of H)13.4 to R1.1 cells. Thus, the $V_LC_9C_{16}$ peptide is a specific Reo3R ligand with a greater ability to inhibit 87.92.6 binding compared with its linear analog.

Inhibition of Reo3R/Reovirus Type 3 Interaction by Peptides—To evaluate further the interaction of the $V_LC_9C_{16}$ peptide with the Reo3R, the peptide was utilized to compete with ^{125}I-reovirus type 3 for binding to the Reo3R. As indicated in Fig. 12, the $V_LC_9C_{16}$ peptide demonstrated greater inhibition of this interaction than the $V_LC_8C_{16}$ or $V_LC_{10}C_{16}$ peptide. When compared with the linear V_L peptide (Fig. 13), the $V_LC_9C_{16}$ peptide demonstrated >40-fold higher inhibitory potency and similar inhibitory potency to the dimeric V_LSH peptide.

Together, Figs. 10–13 indicate that the cyclic and dimeric analogs of the V_L peptide demonstrate greater inhibition of 87.92.6 and reovirus type 3 binding to cellular receptors than the linear peptide analog. This suggests a higher affinity of interaction for these peptides. The optimal conformation for binding appears to be represented by the $V_LC_9C_{16}$ peptide, in agreement with the findings for binding to 9B.G5.

DISCUSSION

The binding of protein molecules to specific receptors is a fundamental aspect of many biological systems. Recombinant DNA technology has allowed analysis of these interactions to be carried to a new level of understanding. The use of site-directed mutagenesis and other related techniques allows analysis of potential contact residues and mapping of potential intermolecular interactions of a variety of receptor ligands. In antibody-receptor systems, much more is known due to the availability of several crystal structures (1, 6, 7), as well as of cocrystallization structures of several antigen-antibody complexes (2–5). These allow prediction of contact residues with a higher degree of probability than in other systems where structural data on the ligand and/or receptor involved is not readily available.

The structural studies of antibody/antigen interactions implicate amino acid side chains from hypervariable regions in forming contacts with antigen. These regions are often reverse turn structures, allowing ready accessibility of the involved side chains to form intermolecular interactions. Whereas the overall surfaces of antibody variable regions are often described as concave in these interactions, some examples exist wherein there is a convex nature to antibody surfaces with

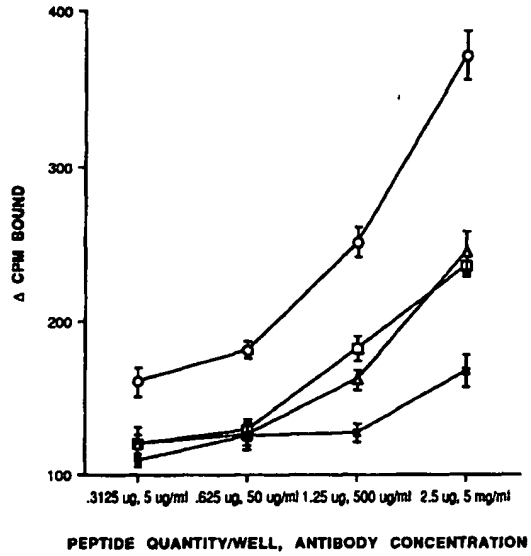

FIG. 5. **Binding of 9B.G5 to peptides on solid-phase RIA.** Peptides in the amounts shown were evaporated onto 96-well RIA plates; the wells were washed and blocked; and binding by 9B.G5 was assessed as described under "Materials and Methods." The counts/minute bound are shown *versus* increasing concentrations of antibody and peptides. The mean ± S.D. of two determinations on replicate samples is shown. □, $V_LC_8C_{16}$; ○, $V_LC_9C_{16}$; △, $V_LC_{10}C_{16}$; ×, control.

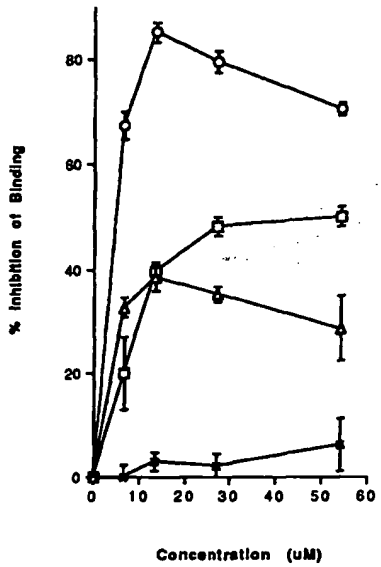

FIG. 6. **Inhibition of 9B.G5/87.92.6 interaction by cyclic peptides.** 9B.G5-coated RIA wells were preincubated with peptides prior to the addition of 87.92.6. The wells were washed, and ^{125}I-labeled goat anti-mouse IgM was added to detect 87.92.6 binding (see "Materials and Methods" for full protocols). Percent inhibition of binding is shown *versus* increasing concentrations of peptides. The mean ± S.E. of two assays is shown. ×, control; □, $V_LC_8C_{16}$; ○, $V_LC_9C_{16}$; △, $V_LC_{10}C_{16}$.

protruberance of specific CDRs. In addition, even where the overall surface is flat or concave, amino acid residues from specific hypervariable loops may form specific interactions central to the energy of binding (8). In these cases, it is possible to develop peptides based on the amino acid sequences of these loops that may display similar properties.

The prior demonstration (10, 14) of the development of bioactive hypervariable loop-derived peptides in the reovirus

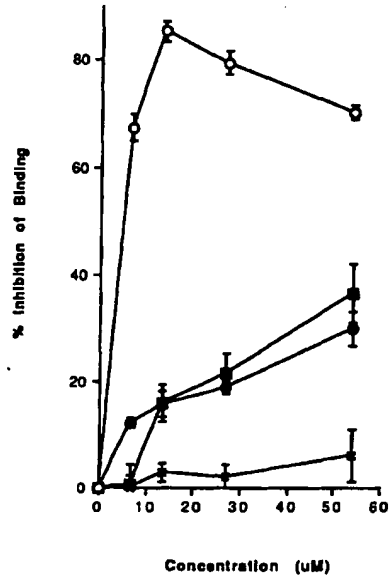

FIG. 7. **Inhibition of 9B.G5/87.92.6 interaction by cyclic peptides.** The protocol is as described for Fig. 6. Comparison with linear peptides derived from the 87.92.6 variable regions is shown for two experiments. ×, control; ■, V_H; ●, V_L; ○, $V_LC_8C_{16}$.

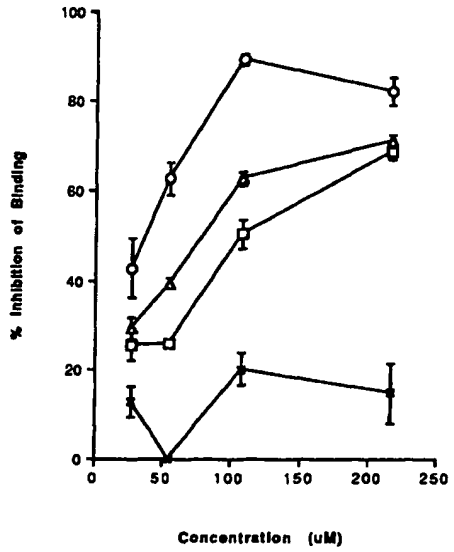

FIG. 8. **Inhibition of 9B.G5/reovirus type 3 interaction by cyclic peptides.** Inhibition of binding of ^{125}I-reovirus type 3 to 9B.G5-coated wells by peptides was determined as described under "Materials and Methods." The mean ± S.E. of two experiments is shown. □, $V_LC_8C_{16}$cyc; ○, $V_LC_9C_{16}$cyc; △, $V_LC_{10}C_{16}$cyc; ×, control.

system was characterized by a low energy of interaction of these peptides. This was likely due, at least in part, to the linear nature of the peptides described. Structural analysis of the predicted binding sites of both HA3 and 87.92.6 indicates a reverse turn structure (10, 24). This structure places the side chains of several critical amino acids (Table I, Tyr-Ser-Gly-Ser-Thr) projecting from the same face of the reverse turn. Prior analysis of peptides wherein the hydroxyl groups of these side chains had been removed indicated that each of them may play a role in receptor interactions.[2] This is supported by preliminary models of the interaction between the V_L peptide and sialic acid, which is one candidate Reo3R utilized by L cells (25, 26).[2] The orientation of these side

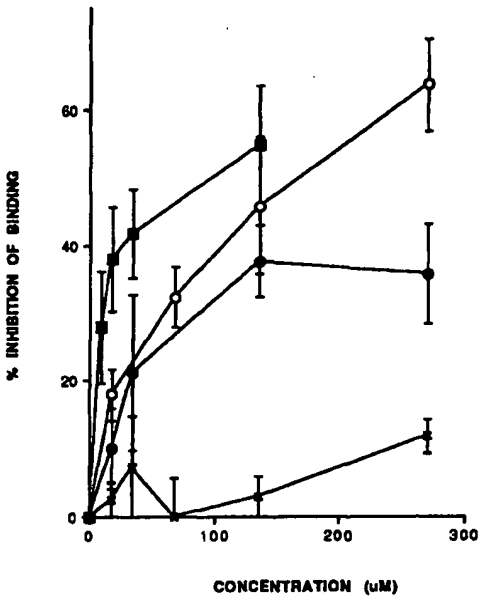

FIG. 9. **Inhibition of 9B.G5/reovirus type 3 interaction by cyclic peptides.** Comparison of linear and dimeric peptides is shown as described for Fig. 8. The mean ± S.E. of two experiments is shown. ■, V_LSH; ○, $V_LC_9C_{16}$; ●, V_L; ×, control.

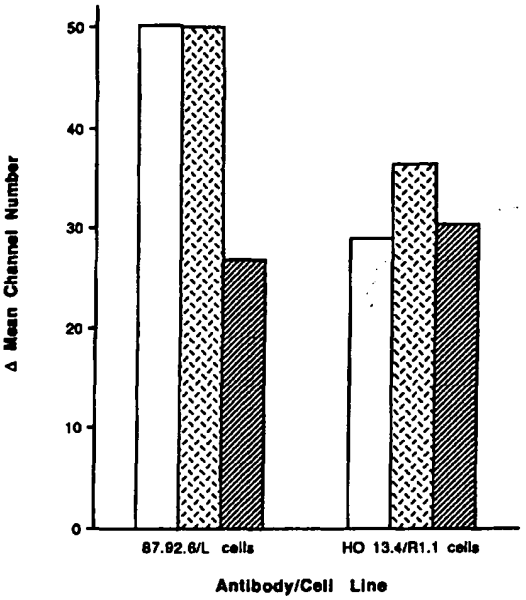

FIG. 11. **Specificity of $V_LC_9C_{16}$ peptide binding to the Reo3R.** The protocol is as described for Fig. 10. The net mean channel number, a function of antibody binding, is shown for cells preincubated with various peptides. *White bar*, control; *dashed bar*, V_H; *hatched bar*, $V_LC_9C_{16}$.

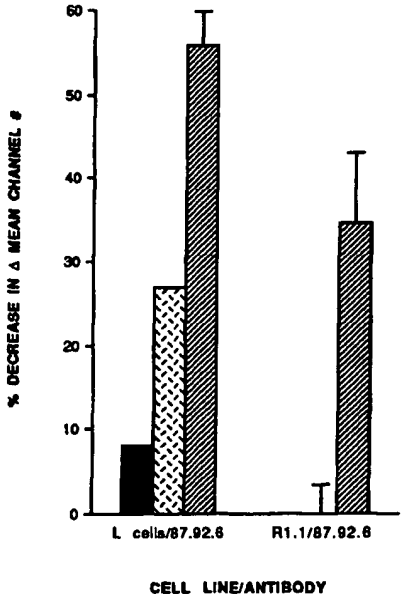

FIG. 10. **Inhibition of Reo3R/87.92.6 interaction by peptides.** Cells were preincubated with peptides prior to addition of antibodies and FACS analysis as described under "Materials and Methods." The mean ± S.E. of two experiments is shown. *Shaded bar*, V_H; *dashed bar*, V_L; *hatched bar*, $V_LC_9C_{16}$.

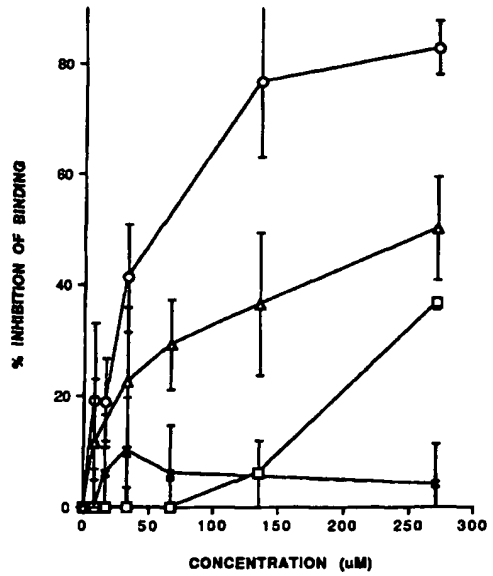

FIG. 12. **Inhibition of Reo3R/reovirus type 3 interaction by peptides.** Murine L cells were preincubated with peptides prior to the addition of ^{125}I-reovirus type 3 and binding analysis as described under "Materials and Methods." Percent inhibition of binding is shown as a function of increasing peptide concentration. The mean ± S.E. of four experiments is shown. ×, control; □, $V_LC_8C_{16}$; ○, $V_LC_9C_{16}$; △, $V_LV_{10}C_{16}$.

chains is critical in forming the appropriate binding geometry. Molecular modeling studies and conformational energy calculations suggest that the sialic acid-binding mode of the V_L peptide is more enthalpically favored than the initial starting geometry or ground state. This would suggest that the linear peptides initially utilized had to overcome the entropy involved in forming the correct binding geometry prior to interacting with the receptor, thereby detracting from the net free energy of binding.

The range of solution comformations available to the linear V_L peptide is not known. However, it appears that the cyclic peptide analogs utilized in this study overcome much of the entropic cost of binding by having a restricted conformation. Thus, even those peptides with suboptimal conformations still demonstrated higher binding to 9B.G5 and the Reo3R than the free V_L peptide (compare Figs. 6 and 7, 8 and 9, and 12 and 13). The $V_LC_9C_{16}$ peptide had the highest binding of all the cyclic analogs tested. This suggests that whereas this

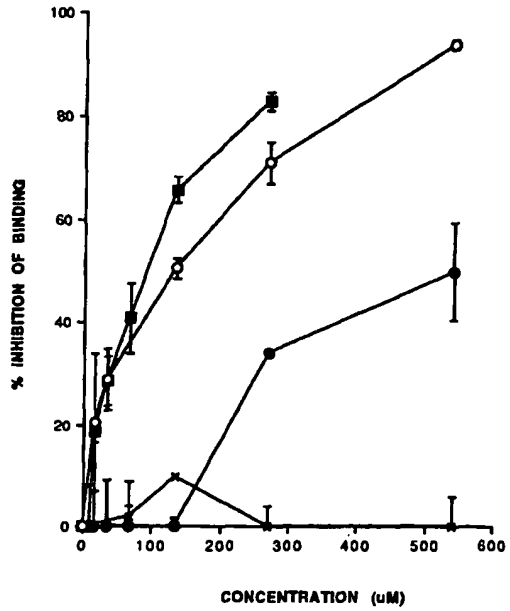

FIG. 13. **Comparison of cyclic and linear peptide interactions with Reo3R by inhibition of ^{125}I-reovirus type 3 binding.** The protocol is as described for Fig. 12. The mean ± S.E. of two experiments is shown. ×, control; ●, V_L; ■, V_LSH; ○, $V_LC_9C_{16}$.

peptide is not the closest in backbone configuration to the previous model structures of this epitope (root mean square of 1.52 for $V_LC_9C_{16}$ *versus* 0.96 for $V_LV_{10}C_{16}$), this peptide most readily assumes the binding mode. The implications of this finding with regard to interactions with sialic acid are being evaluated.

The higher binding of the dimeric V_LSH peptide compared with the V_L monomer is likely due, at least in part, to increased avidity, although conformational stabilization is also possible. However, this dimeric peptide is also able to down-modulate the Reo3R on cells and induce growth inhibition (14), alter Schwann cell growth and function (19), and change the growth and differentiation of oligodendrocytes (20). When tested *in vivo*, this peptide also induces demyelination of rat optic nerves.[3] None of these effects were seen with monomeric forms of the V_L peptide. This is likely due to the ability of the dimeric peptide to induce receptor cross-linking. Thus, increasing avidity by dimerization, although effective in this system, also may impart biological activities to similarly designed peptides that need to be carefully evaluated.

In addition to antibody molecules, other receptor ligands may employ binding strategies similar to those of antibody molecules. The CDR loops of antibodies usually are reverse turn or helix-like structures. Other proteins that demonstrate specific binding characteristics for receptor structures (including biological receptors, enzymes, and carrier proteins) also frequently may employ similar reverse turn or helix-like structures, and these may be similar to antibody CDRs (28). By performing structural analysis of these sites, cyclic peptides can be developed similar to those noted above.

Together, these results indicate that cyclic peptide analogs developed by structural analysis and molecular modeling based on antibody structures can allow analysis of the binding mode of individual hypervariable loop-derived structures. Such cyclic peptides may more accurately mimic the binding conformation than linear analogs and can demonstrate enhanced biological activity. These observations may be of utility for drug design and pharmaceutical development. Cyclic peptide analogs of other receptor ligands have been developed (29–32) and have been of utility in the analysis of various aspects of receptor binding. Hypervariable loop sequences from other biologically active antibody structures can be utilized to generate synthetic peptide analogs that may possess similar properties. By optimizing the conformation of such peptides by cyclization as reported here, the affinity and specificity of such peptides may be improved. In addition, similar cyclic peptides with biological activity may be derived from analyses of protein structures with similarities to antibody hypervariable regions. Many chemical strategies for cyclization have been developed by several laboratories (examples include Refs. 15, 27), and similar synthetic routes may be of utility in developing biostable peptides.

There are currently cyclic peptides in clinical use, such as cyclosporin. These peptides display marked biostability when administered *in vivo*, when compared with linear peptides. This may be due in part to the cyclic nature of these peptides. One of the major problems encountered in the clinical use of linear peptides is their susceptibility to degradation by proteolytic enzymes and peptidases. The degradation of these linear peptides may proceed from the free amino or carboxyl terminus. Cyclic peptides such as cyclosporin do not possess a free amino or carboxyl terminus, and this may contribute to their stability *in vivo*.

Thus, hypervariable region-based cyclic peptides and conformationally constrained peptides may be developed to possess enhanced biostability as well as enhanced affinity. The synthetic route for development of such cyclic peptides has recently been elucidated, and several examples of synthetic routes for the development of both cyclic and conformationally constrained peptides can be found in the literature. Such peptides can be developed to mimic both reverse turn structures as well as helix-like structures. Development of biologically active cyclic peptides directly from amino acid sequence information of antibodies and antibody-like structures could lead to the development of a novel group of therapeutic and biologically active agents.

Acknowledgment—We would like to thank J. Paul for his helpful comments.

[3] J. Cohen, W. Williams, D. Weiner, and M. Greene, submitted for publication.

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Antibody engineering: the use of *Escherichia coli* as an expression host

E. SALLY WARD
Cancer Immunobiology Center and Department of Microbiology, The University of Texas Southwestern Medical Center, Dallas, Texas 75235-9048, USA

ABSTRACT **The hypervariable loops of an antibody molecule are supported on the relatively conserved β-sheeted frameworks of the heavy- and light-chain variable domains (designated VH and VL domains, respectively). Residues within and flanking these loops interact with antigen and confer the specificity and affinity of antigen binding on the immunoglobulin molecule. Thus, the isolation and expression of VH and VL domain genes are of particular interest both for analysis of the determinants of antibody specificity and for generation of fragments with binding affinities for use in therapy and diagnosis. The PCR can now be used to isolate diverse repertoires of antibody VH and VL domain genes from antibody-producing cells from different species, including humans and mice. The genes can be expressed as either secreted or surface-bound Fv or Fab fragments, using *Escherichia coli* expression systems, and the desired antigen-binding specificity screened for or, preferably, selected. The use of *E. coli* as an expression host allows the required antigen-binding specificity to be isolated in clonal form in a matter of days. The VH and VL domain genes can also be hypermutated and higher-affinity variants isolated by screening or selection. Thus, the use of this technology should allow the isolation of novel binding specificities or specificities that are difficult to generate by hybridoma technology. It will also facilitate the isolation of human-derived Fv/Fab fragments that may be less immunogenic in therapy. This approach therefore has almost unlimited potential in the generation of therapeutics with binding specificities to order. The fragments can be used either alone or linked to effector functions in the form of antibody-constant domains or toxins. The new technology could prove to be a method of choice for the rapid and convenient production of designer antibodies.—Ward, E. S. Antibody engineering: the use of *Escherichia coli* as an expression host. *FASEB J.* 6: 2422-2427; 1992.**

Key Words: VH and VL genes • polymerase chain reaction • recombinant antibody fragments • bacteriophage surface expression

THE DEVELOPMENT OF HYBRIDOMA TECHNOLOGY (1) in the middle 1970s has resulted in an enormous expansion of the use of antibodies in medicine and biology. This technology allows the production of antibodies of defined specificity in clonal form. More recently, developments in genetic manipulation facilitate the isolation of diverse repertoires of antibody variable-domain genes from immunoglobulin-producing cells followed by their expression using *Escherichia coli* as a host (2-4). Thus, the polymerase chain reaction (PCR)[1] (5) can be routinely applied to the isolation of diverse repertoires of antibody genes from different species, including mouse and human (2-4, 6, 7). These repertoires can be cloned in *E. coli* for expression, and recombinant clones producing immunoglobulin fragments with desired binding specificities screened for (2, 4, 8-10) or, more recently, selected for (11, 12). This review will focus on these recent developments and discuss possible applications of the new technology in the production of engineered antibodies for therapy and diagnosis.

USE OF THE POLYMERASE CHAIN REACTION TO ISOLATE IMMUNOGLOBULIN GENES

The PCR (5) can be used to isolate individual genes and members of gene families; the only requirement is some preexisting knowledge of the sequences of either the gene (by protein sequencing, for example) or members of the family. The sequence knowledge does not have to be complete, as the PCR can be used with partially degenerate oligonucleotide primers. For the immunoglobulin variable-domain sequences, databases exist that document the known sequences for different species. For example, the Kabat database (13) contains sequence information for immunoglobulin genes for nine species, including human, mouse, rat, and rabbit. Initially, a comparison of the nucleotide sequences of murine immunoglobulin variable-domain genes was used to design universal oligonucleotide primers that anneal to the 5' and 3' ends of antibody heavy-chain variable (VH) and light-chain variable (VL) domain genes (**Fig. 1**). Moreover, these primers were designed with internal restriction sites, which allows the forced cloning of the PCR products directly for expression (6). Alternatively, for human and mouse VH and VL genes, PCR primers have been designed that are specific for different VH and VL families at the 5' ends and/or anneal to the highly conserved constant-domain genes (4, 7, 14). A further approach is to design primers that are complementary to the leader sequences rather than the 5' ends of the mature VH or VL genes (15-17) (Fig. 1).

Thus, a variety of primers exist that can be used in the PCR to isolate either diverse repertoires of antibody variable-domain genes from heterogeneous populations of antibody-producing cells or, more simply, the variable-domain genes from a particular hybridoma. The repertoires of VH and VL genes can be cloned for expression in *E. coli* as VH domains (2), Fv fragments (2, 18), single-chain Fv (scFv) fragments (19, 20), or Fab fragments (21) (**Fig. 2**). The challenges now lie in the development of systems for the screening, and preferably selection, of clones producing antibody fragments of the desired antigen-binding specificity.

[1]Abbreviations: PCR, polymerase chain reaction; scFv, single-chain Fv; CDR, complementarity-determining region; SOE, splicing by overlap extension; phOx, 2-phenyloxazol-5-one; ADCC, antibody-dependent cell-mediated cytotoxicity; VH, heavy-chain variable; VL, light-chain variable.

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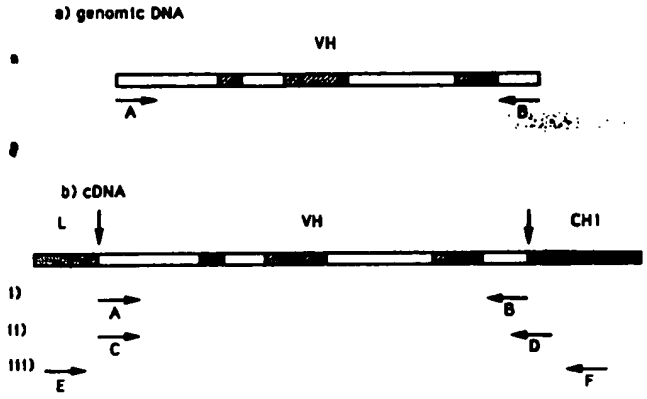

Figure 1. Strategies for PCR amplification of immunoglobulin VH domain genes: *a*) from genomic DNA isolated from antibody-producing cells, primers A (5') and B (3') can be used to isolate the rearranged VH genes (2, 6). These primers anneal to the 5' and 3' ends of the coding sequences of the VH domain genes, and productively and nonproductively rearranged genes will both be isolated; *b*) from cDNA isolated from antibody-producing cells. *i*) primers A (5') and B (3') can be used, as in *a*. In this case, only productively rearranged genes will be isolated; *ii*) primers C (5') and D (3') can be used on cDNA only, as primer D overlaps both the J region and CH1 domain (7); *iii*) primers E (5') and F (3') can be used on cDNA only, because E anneals to the leader sequence (L) and F anneals to the CH1 domain (15–17). All primers can be partially degenerate or family-specific. Hatched boxes represent CDR1, CDR2, and CDR3. The leader exon is represented by a stippled box, and the 5' end of the CH1 domain by horizontal hatching. Vertical arrows indicate the location of intron-exon boundaries in the corresponding genomic DNA that encodes rearranged immunoglobulin genes. Similar strategies can be used for the isolation of VL genes.

EXPRESSION OF IMMUNOGLOBULIN FRAGMENTS IN *E. COLI*

The development of secretion systems for the production of functional Fv and Fab fragments (18, 21) has led to the use of *E. coli* as a host of choice for the expression of immunoglobulin fragments. In these systems, signal sequences such as the pectate lyase (pelB) leader sequence (21), which is derived from the pelB gene of *Erwinia carotovora*, are linked up in translational frame to the genes encoding immunoglobulin fragments. This results in secretion of the expressed protein into the periplasmic space. Fv fragments can be secreted in yields of 2–10 mg/l of culture and Fabs in yields of 2–5 mg/l (2, 18, 21). Although Fv fragments may have advantages over Fabs due to their small size, the stability of the noncovalent VH-VL domain association appears to be variable. This probably results from differences in the sequences of the third hypervariable (CDR3) loops from one antibody to another. These loops form the core of the antigen-binding site that bridges the interface of the VH-VL domain interaction (23–25). These residues may, if not directly involved, affect the stability of the VH-VL domain interaction by modulation of the conformation of the flanking framework residues.

There are several ways of stabilizing the association, as follows.

1) The VH and VL domains can be expressed as a scFv fragment (19, 20, 26), in which the VH domain is linked by a peptide linker to the VL domain. Suitable peptide linkers have now been designed, and the scFvs can be expressed as either intracellular inclusion bodies (19, 20, 26) or as secreted proteins (A. D. Griffiths and E. S. Ward, unpublished

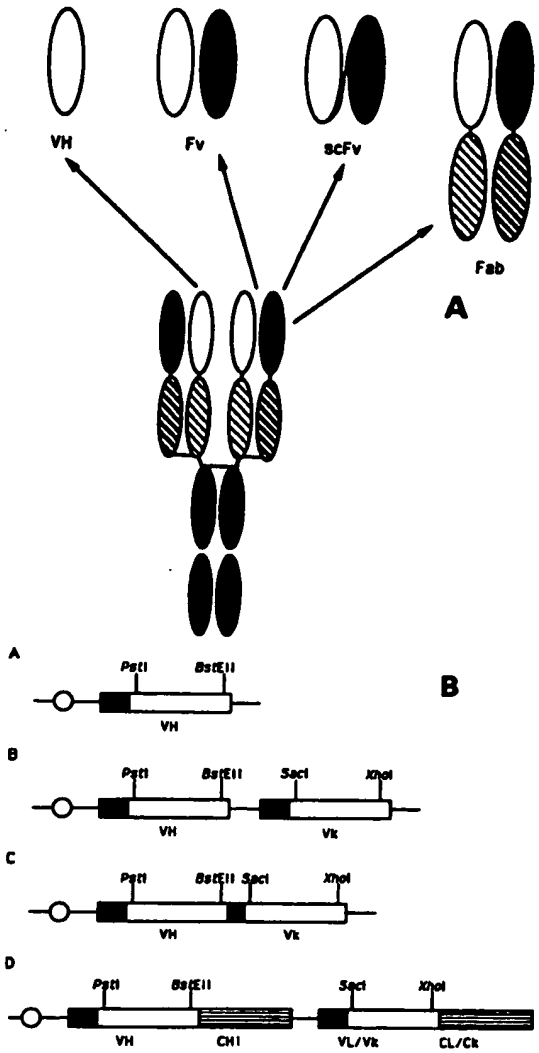

Figure 2A. Schematic representation of the immunoglobulin molecule. The immunoglobulin IgG molecule consists of strings of discrete domains, and comprises two heavy chains and two light chains (22). The heavy and light chains are linked by an intermolecular disulfide bridge (indicated by a horizontal line between the hatched CH1 and CL domains). The heavy chains are also linked to each other by one or more intermolecular disulfide bridges. These -S-S- bridges are in the hinge region, which links the CH1 domain to the CH2 domain (indicated by horizontal hatching). The CH3 domain is indicated by vertical hatching. Immunoglobulin-derived fragments that can be expressed and secreted from recombinant *E. coli* cells are shown at the top of the figure. VH, heavy-chain variable domain; Fv, VH and light-chain variable (VL) domains; scFv, VH and VL domains linked by a synthetic peptide linker (19, 20); Fab, Fd (VH linked to CH1 domain) and paired light chain. *B*) Plasmid vectors for the secretion of immunoglobulin fragments (2, 4). A, VH domains; B, Fv fragments; C, scFv fragments, and D, Fab fragments. The restriction sites in these plasmids are designed for the cloning of murine VH and VL genes, and can be modified to accommodate genes tailored with different restriction sites. VH genes are cloned as *PstI-BstEII* fragments, and the VL genes as *SacI-XhoI* fragments. These sites are incorporated during the PCR (primers for VH genes are as described in refs 2, 6; for VL genes, VK2BACK, ref 38, and 3' primer: 5'-CCG TTT CAG CTC GAG CTT GGT CCC 3'). The pelB leader sequences (21) are represented by stippled boxes, VH and VL genes by open boxes, the single-chain linker sequence (20) by vertical hatching, and the antibody CH1 and CL domains by horizontal hatching. Open circles represent the *lacz* promoter.

results). The secretion yields may be lower than those using resolubilization from isolated inclusion bodies, but the lower yields may be offset by the relative ease of purification by using a secretion system. If high yields are desirable, for example in industrial scaleup, the isolation from inclusion bodies may be a preferable route. In some cases, a small decrease in binding affinity has been observed for scFvs compared with the corresponding Fv (19, 20), but generally this is not significant. For scFvs isolated from inclusion bodies, the decrease in affinity may be due to incomplete renaturation rather than the presence of the single chain linker.

2) Genetic engineering has been used to insert an intramolecular -S-S- bridge between the two domains (light-chain residue 55 and heavy-chain residue 108; light-chain residue 56 and heavy-chain residue 106) (27). This results in the expression of functionally active Fv fragments that are stably associated.

3) Glutaraldehyde treatment has been used to chemically cross-link the VH and VL domains (27).

ANTIBODY FRAGMENTS WITH ANTIGEN-BINDING ACTIVITIES

Diverse repertoires of antibody VH domain genes, isolated by the PCR, have been cloned for expression in *E. coli* using the pelB leader sequence to direct the expressed protein into the periplasmic space (2). After several hours of induction, the expressed protein starts to leak from the periplasmic space into the culture supernatant (18). Culture supernatants can therefore be conveniently screened for the presence of fragments with antigen-binding specificities by ELISA. Using such a secretion system, VH domains with antigen-binding activities were isolated against two different antigens (lysozyme and keyhole limpet hemocyanin) from repertoires of VH domain genes generated by the PCR from the spleen DNA of immunized mice (2). The anti-lysozyme VH domains were characterized and shown to have high and specific antigen-binding affinities. Subsequently, VH domains with specificities against mucin and influenza virus neuraminidase have been isolated (D. Allen, personal communication; P. Hudson, personal communication).

The rather hydrophobic nature of VH domains, due to the exposed residues that are normally capped off by the paired light chain (28), may offset the advantages of their small size. It may be possible to reduce the hydrophobicity by judicious replacement of the hydrophobic residues by more hydrophilic ones using protein engineering, which would make these small domains attractive reagents for use in therapy and diagnosis.

The smallest units of antigen binding isolated to date are complementarity-determining region (CDR)-derived peptides (29, 30). These peptides were designed on the basis of modeling and/or sequence analysis, and could, if a general feature for a larger number of antibodies, prove to have potential uses in therapy— for example, as blocking reagents.

RANDOM COMBINATIONS OF REPERTOIRES OF VH AND VL DOMAIN GENES

An alternative approach for the isolation of fragments with antigen-binding specificities from repertoires is to randomly combine the isolated VH and VL genes by either restriction at a unique site (4), or by using splicing by overlap extension (SOE, ref 31, Fig. 3). Huse and colleagues (4) reported the expression of randomly combined variable-domain genes as Fab fragments using a lambda Zap expression vector. After

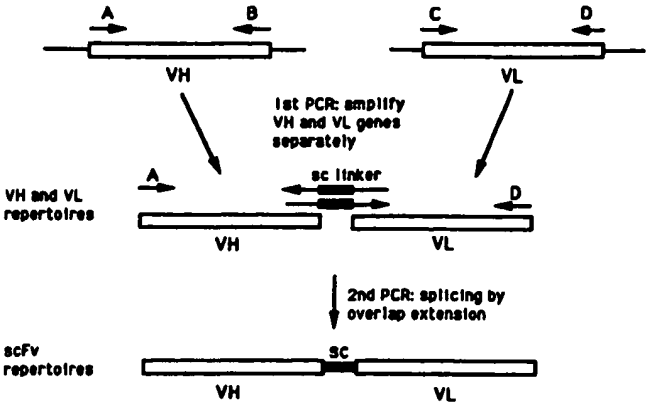

Figure 3. Schematic representation of splicing by overlap extension (SOE, ref 31) for the generation of scFv genes from random combinations of repertoires of VH and VL domain genes (38). The VH genes are isolated from antibody-producing cells using the PCR and primers A (5') and B (3'). The VL genes are isolated from antibody-producing cells in a separate PCR using primers C (5') and D (3'). The VH and VL gene repertoires are randomly combined by splicing together in a PCR with primers A and D, together with single chain (sc) linker primers. These sc linker primers encode a synthetic peptide linker (20) and also have regions of identity or complementarity to primers B (3' of VH genes) and C (5' of VL genes). Open boxes represent the VH and VL domain genes, and stippled boxes represent the sc linker sequence.

transfection, the resulting plaques were screened using radiolabeled antigen. Initially this was carried out for the hapten NPN, and has since been extended to the isolation of antigen-binding activities from repertoires of genes derived from both murine and human antibody-producing cells (8–10). For example, this approach has been used to isolate anti-tetanus toxoid Fabs from repertoires derived from PBLs from humans (who have recently been boosted against tetanus infection), demonstrating the utility of this route for the isolation of 'pure' human antibodies (9, 10). This is significant, as to date the production of human monoclonal antibodies by hybridoma technology has proved to be difficult due to both technical and ethical barriers (32–35). A general problem encountered during the isolation of human antibodies is that for ethical reasons it is not possible to immunize humans. Without immunization the frequency of antigen-specific clones will probably be extremely low, as exposure of the immune system to immunogen results in clonal expansion and affinity maturation of antigen-specific cells with extremely high efficiency (36, 37). Thus, to isolate fragments with antigen-binding specificities from naive repertoires, using *E. coli* as a host, it is necessary to mimic the efficiency of the in vivo immune system in identifying low-frequency, and possibly low-affinity, antigen-specific clones. Thus, the challenges lie in the development of suitable selection systems that will allow this goal to be achieved.

SELECTION VS. SCREENING

The systems described previously involve screening rather than selection. To isolate low-frequency clones from gene repertoires derived from unimmunized animals there is a clear need for the development of selection systems to avoid tedious screening. McCafferty and colleagues (11) have designed such a selection system that involves the expression of

scFvs on the surface of bacteriophage fd. This is achieved by insertion of genes encoding scFv fragments into the gene III coat protein of the bacteriophage. Initially, the anti-lysozyme D1.3 scFv was expressed in this way, and phage expressing antigen-binding activities enriched for, by passage over lysozyme sepharose, by a factor of a million from a pool of irrelevant phage. This system has more recently been modified and extended to the selection of scFvs with anti-2-phenyloxazol-5-one (phOx) binding activities, with a range of affinities, from repertoires of VH and VL domain genes derived from spleen cDNA of immunized mice (38). For combinatorial libraries (of about 10^6 clones in size) derived from unimmunized animals, no phOx binding activities were selected (38). This suggests that to isolate binding activities from unimmunized repertoires, it will be necessary to generate extremely large libraries.

A similar phage surface expression system has been described by Kang and colleagues (12), who expressed Fab fragments on the surface of bacteriophage M13 by linkage to the gene VIII coat protein. This system differs from the fd system insofar as there are 1–24 copies of the gene VIII protein that are expressed over the entire phage surface. In contrast, the gene III coat protein is expressed in three or four copies per phage particle and these are located at the tip of the particle.

The phage systems (11, 12) should prove to be extremely useful tools for the selection of antigen-binding activities from repertoires. Alternatively, these selection systems could be applied to the generation of higher-affinity antibodies. After identification of phage with surface-bound binding activities, it is easy to isolate the VH and VL genes by PCR and clone them for secretion from *E. coli* as either (sc)Fv or Fab fragments, using vectors similar to those shown in Fig. 2*B*. This allows the selection of fragments of the desired specificity to be followed by purification and characterization.

Thus, there are now ways to isolate antibody variable-domain genes by the PCR, to clone these for expression and to select phage which bear fragments with antigen-binding affinities (Fig. 4). After isolation of the Fv or Fab of desired binding specificity, there are many potential uses. It may be desirable to increase the affinity of the fragments using random PCR mutagenesis (39) followed by selection (11, 12). The high-affinity fragments may have uses either alone or linked up genetically to effector functions to generate reagents for therapy and diagnosis.

GENERATION OF HIGHER-AFFINITY ANTIBODIES BY IN VITRO MUTAGENESIS

Random mutagenesis can be used, coupled with the PCR, to hypermutate particular regions of an antibody-variable domain. This has been applied to the hypermutation of the anti-lysozyme D1.3 VH domain, and higher-affinity variants have been isolated by tedious screening (39). The phage selection systems now offer an attractive alternative for the selection of higher-affinity variants from many hypermutated clones. This could prove to be an extremely useful route for improvement of the binding affinities of antibody fragments. It may even be possible to hypermutate a germline variable-domain gene and generate novel specificities, and this could be particularly useful when the antigen is not immunogenic, or for the production and isolation of human-derived antibodies for which immunization is not ethical. Questions arise as to how much hypermutation of a human VH or VL domain can be carried out before the immunoglobulin fragments become immunogenic, if they are to be used in human therapy. For example, will hypermutation of CDR loops result in sequence motifs that are antigenic in vivo? In addition, will such new specificities have autoreactivities; in other words, will such novel specificities represent self-reactive clones that are normally forbidden in vivo?

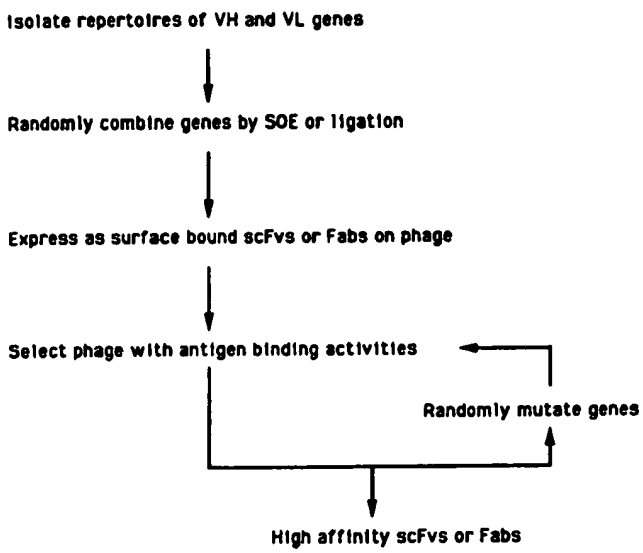

Figure 4. Scheme for the production of high-affinity Fv or Fab fragments using a combination of bacteriophage selection systems (11, 12, 38) and random mutagenesis. For further details, see text.

USES OF IMMUNOGLOBULIN FRAGMENTS FOR IN VIVO THERAPY AND DIAGNOSIS

Immunoglobulin Fv and Fab fragments have been demonstrated to have rapid clearance rates in vivo, with half-lives on the order of 10–60 min (40; A. J. Cumber, E. S. Ward, G. Winter, G. D. Parnell, and E. J. Wawrzynczak, unpublished results). The absence of the immunoglobulin CH2 domain (41), together with the small size of the fragments, are probably the main factors that result in the short half-lives. In this respect, an $F(ab)_2$ fragment has been shown to have a half-life approximately twice as long as that of the corresponding Fab fragment (40). Fv fragments may also have a tendency to dissociate in vivo, as recently demonstrated for the anti-lysozyme D1.3 Fv (A. J. Cumber et al., unpublished results), and scFv or Fab fragments may therefore be preferable reagents for in vivo applications. Alternatively, a combination of genetic engineering and protein chemistry have been used to construct a bivalent Fv fragment that has improved stability in vivo (A. J. Cumber et al., unpublished results), and the bivalent nature of this protein may be advantageous as it increases the avidity of the interaction with antigen.

Immunoglobulin fragments have uses in clinical situations where rapid clearance is either required or advantageous. Such a situation is the imaging of tumors, as the rapid clearance results in reduced immunogenicity. This is of particular relevance if the imaging is to be followed by therapy with the same antibody as a complete molecule. In addition, Fabs have been shown to give high tumor: normal tissue ratios compared with complete antibodies (42), possibly due to the lack of Fc receptor-mediated binding (43, 44) and rapid clearance. A further application of immunoglobulin fragments is for the treatment of drug overdoses, as they could

be used to 'mop up' the excess drug and clear it rapidly from the circulation.

REBUILDING FV AND FAB FRAGMENTS INTO REAGENTS WITH EFFECTOR FUNCTIONS

For treatment of disease (for example, cancer or infections) there is a need to graft effector functions onto the antibody fragments, such as toxins (45–49) or the Fc portion of the immunoglobulin molecule (43, 44, 50). A truncated form of *Pseudomonas* exotoxin has been genetically linked to an antitumor Fv fragment to generate an immunotoxin that can be expressed in high yields in *E. coli* (48). In addition, an Fab-toxin construction, containing the gene encoding phospholipase C from *Clostridium perfringens*, has been secreted in functional form from recombinant *E. coli* cells (49). A limitation of Fv-toxin conjugates may be that they are highly immunogenic due to the toxin moiety and also have a rapid rate of clearance. It may be possible to avoid an undesirable immune response by using different toxin moieties for cases where repeated doses are necessary. The feasibility of this clearly depends on the availability of different Fv-toxin conjugates with suitable properties for therapy.

For some situations therefore it may be preferable to use the Fv or Fab fragments of desired specificity as building blocks for complete immunoglobulin molecules. The complete immunoglobulin is stable in vivo (half-life of days), and providing the isotype is derived from the same species, it is less immunogenic than toxin moieties. The choice of isotype will depend on the functions required, as some studies have indicated that different isotypes vary considerably in their complement lytic and antibody-dependent cell-mediated cytotoxicity (ADCC) activities (51). It appears that for most clinical situations, the IgG1 isotype is the one of choice (51). This isotype has been used effectively in therapy, linked for example to the humanized variable domains of CAMPATH-I (52). After rebuilding of an Fv or Fab fragment into a complete antibody at the genetic level, the antibody can be expressed using either mammalian cell transfectomas (53) or the baculovirus system (54), which has recently been shown to express high yields of functionally active antibodies. Thus, antibody fragments can be rapidly isolated by selection from VH and VL domain repertoires which are expressed on the surface of bacteriophage, and used to rebuild antibodies for therapy. Such tailor-made antibodies have the advantages of high affinity and specificity (if necessary generated by in vitro mutagenesis), and the effector functions of choice. These recombinant antibodies promise to have extensive uses in therapy and diagnosis. FJ

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Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli.

Ward ES, Gussow D, Griffiths AD, Jones PT, Winter G.

MRC Laboratory of Molecular Biology, Cambridge, UK.

In antibodies, a heavy and a light chain variable domain, VH and VL, respectively, pack together and the hypervariable loops on each domain contribute to binding antigen. We find, however, that isolated VH domains with good antigen-binding affinities can also be prepared. Using the polymerase chain reaction, diverse libraries of VH genes were cloned from the spleen genomic DNA of mice immunized with either lysozyme or keyhole-limpet haemocyanin. From these libraries, VH domains were expressed and secreted from Escherichia coli. Binding activities were detected against both antigens, and two VH domains were characterized with affinities for lysozyme in the 20 nM range. Isolated variable domains may offer an alternative to monoclonal antibodies and serve as the key to building high-affinity human antibodies. We suggest the name 'single domain antibodies (dAbs)' for these antigen binding demands.

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